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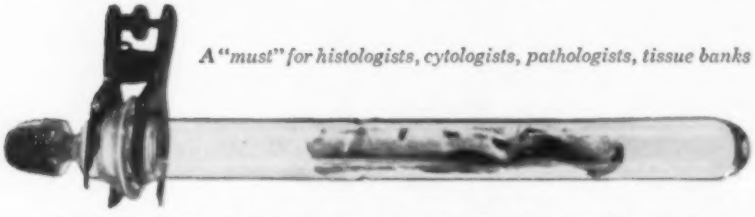

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# 1958 Iodine Research Award Nominations Requested Prior to January 1, 1958

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1958 Chilean Iodine Educational Bureau, Inc., Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1958 Award, *nominations must be received on or before January 1, 1958.*

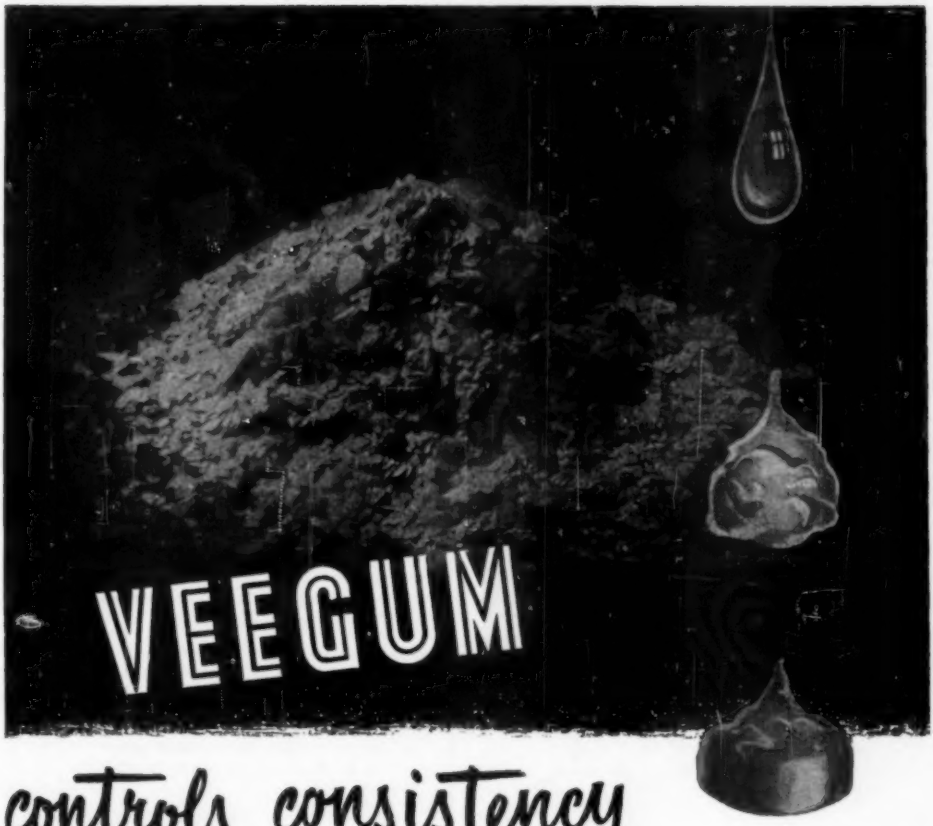
A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; F. F. Blicke, E. A. Brecht, John E. Christian, Stanley G. Mittelstaedt, Lloyd M. Parks, and Linwood F. Tice.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its tenth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn, received the sixth award and Dr. Dominick Papa, Schering Corporation, Bloomfield, N. J., received the seventh award. The eighth award went to Dr. John E. Christian, Professor of Pharmaceutical Chemistry, School of Pharmacy, Purdue University, Lafayette, Indiana. The ninth award was conferred on Dr. James O. Hoppe, Research Pharmacologist, Sterling-Winthrop Research Institute, Rensselear, New York, during the 1957 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

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The next annual meeting of the American Pharmaceutical Association will be held in Los Angeles, California, April 20 to 26, 1958. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than February 1, 1958, or preferably earlier, in order to assure a place on any Section program.

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLVI — DECEMBER 1957 — NUMBER 12 — CONSECUTIVE NO. 24

# A Comparison of *In vivo* and *In vitro* Tests for the Absorption, Penetration, and Diffusion of Some Medicinals from Silicone and Petrolatum Ointment Bases*

By JOY BICKMORE PLEIN and ELMER M. PLEIN

**Five drugs—sulfanilamide, ammoniated mercury, salicylic acid, iodine and sodium radio-iodide ($I^{131}$), and chlortetracycline—each incorporated into three silicone ointment bases and three petrolatum ointment bases, were tested by *in vitro* procedures for diffusion of the drug and by *in vivo* procedures for penetration and absorption of the drug. A modified agar plate method and a chemical procedure were the two *in vitro* tests used for measuring diffusion. The penetration of the drugs from ointments was determined by applying the ointments to the skin of white rats and subsequently analyzing a biopsy of the inuncted skin. The absorption of the drugs into systemic circulation was measured by analyzing the blood or a "storage organ" for the drugs. Both intact-skin studies and abraded-skin studies were conducted. The results obtained by *in vitro* diffusion tests did not correlate well with the penetration of the drugs as shown by *in vivo* tests. With three of the five drugs the *in vitro* data correlated fairly well with the absorption through abraded skin; whereas there was little correlation between *in vitro* data and absorption through intact skin. Neither the silicone ointment bases nor the petrolatum ointment bases could be shown to be the universally more efficient vehicles.**

IN ORDER FOR THE PATIENT to obtain optimum therapeutic effect from topically applied medication and also, in order to avoid potential toxicity from systemic absorption of drugs applied topically, it is important that the efficiency of the vehicle in allowing or promoting the diffusion, penetration, and absorption of drugs incorporated in it be determined. A comprehensive survey of the literature shows that there have been a great many *in vitro* investigations of the diffusion of drugs from ointments and many *in vivo* tests of percutaneous penetration and absorption of drugs from topical vehicles; however, the data which have been obtained by the many investigators are not conclusive in regard to a correlation between the results gained by *in vitro* and *in vivo* testing and there have been few experiments designed

* Received March 11, 1957, from the College of Pharmacy, University of Washington, Seattle.
Presented to the Scientific Section, A. PH. A., Detroit meeting, April 1956.
Abstracted from a thesis submitted to the Graduate School of the University of Washington by Ellen Joy Plein in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
This research was in part, supported by the State of Washington Funds for Research in Biology and Medicine and by the Research Fund of the Graduate School.
The silicone oils were supplied by Dow Corning Corp. Lederle Laboratories Division of American Cyanamid Co. supplied the chlortetracycline hydrochloride.

with the primary purpose of evaluating the testing methods themselves.

The primary objectives in this study were to determine whether or not the results of *in vitro* laboratory procedures for the determination of diffusion of drugs from ointments would correlate with the results of *in vivo* testing of penetration and absorption of drugs from ointments; and also, to determine whether or not there was a difference in the diffusion, penetration, and absorption of drugs from silicone ointments and from petrolatum ointments. The development of new testing methods or improvement of present procedures was also an interest in this investigation.

As used in this study "diffusion" refers to the passive transfer of the drug from the ointment base into the test medium. *In vitro* methods may be used to study diffusion, since the complex subject of the passage of the drug across the living cellular membrane is not involved. "Penetration" refers to the ability of the drug to enter the skin itself, and "absorption" denotes passage of the drug through the skin and into systemic circulation. Penetration and absorption must, of course, be studied by *in vivo* techniques.

## EXPERIMENTAL

The general plan for the experimental work was to select a group of drugs for which tests could be conducted in order to determine their diffusion, penetration, and absorption; to incorporate these drugs into silicone oil ointment bases and petrolatum ointment bases; and then to test each drug in each ointment base by both *in vitro* and *in vivo* techniques.

A modified agar plate method and a chemical procedure were the two *in vitro* tests used for measuring diffusion. The penetration of the drugs from ointments was determined by applying the medicated ointments to the skin of white rats and subsequently analyzing quantitatively a biopsy of the inuncted skin. The absorption of the drug into systemic circulation was measured by analyzing the blood or a storage organ for the specific drug. Determination of penetration into the skin and of systemic absorption were conducted concurrently, so that the same animals could be used for both tests, and each ointment was tested on animals whose skin was intact and animals whose skin had been abraded.

The five drugs selected were sulfanilamide, ammoniated mercury, salicylic acid, iodine and sodium radio-iodide ($I^{131}$),[1] and chlortetracycline.

Throughout the study significance of differences in the means of two groups of data was analyzed statistically by use of the Student *t*-test and between three or more groups of data by the analysis of variance (1). Differences in which $P = 0.010$ or in which $P < 0.010$ were considered significant differences.

[1] It has been shown that topically applied iodine is converted to the iodide during absorption (2). Iodine was, therefore, used in the diffusion studies and these results were compared with absorption and penetration studies utilizing sodium radio-iodide ($I^{131}$).

**Formulation of Ointment Bases and Medicated Ointments.**—Silicone ointment bases and petrolatum ointment bases which were identical in formula except for the oils used were prepared. The formulas for these ointment bases follow:

| | |
|---|---|
| **1. Simple Petrolatum Ointment** | |
| Synthetic Japan Wax[2] | 20.0 |
| Liquid Petrolatum | 80.0 |
| | 100.0 |
| **2. Simple Silicone Ointment** | |
| Synthetic Japan Wax | 20.0 |
| DC 200, 350 cts.[3] | 80.0 |
| | 100.0 |
| **3. Petrolatum Absorption Base** | |
| Arlacel C | 6.0 |
| Synthetic Japan Wax | 10.0 |
| Liquid Petrolatum | 50.0 |
| White Petrolatum | 15.0 |
| Wool Fat | 10.0 |
| | 100.0 |
| **4. Silicone Absorption Base** | |
| Arlacel C | 6.0 |
| Synthetic Japan Wax | 10.0 |
| DC 200, 350 cts. | 74.0 |
| Wool Fat | 10.0 |
| | 100.0 |
| **5. Petrolatum Emulsion Base** | |
| Cetyl Alcohol | 10.0 |
| Liquid Petrolatum | 20.0 |
| White Petrolatum | 5.0 |
| Sodium Lauryl Sulfate | 1.0 |
| Distilled Water | 64.0 |
| Methylparaben | 0.025 |
| Propylparaben | 0.015 |
| | 100.040 |
| **6. Silicone Emulsion Base** | |
| Cetyl Alcohol | 10.0 |
| DC 200, 1000 cts. | 25.0 |
| Sodium Lauryl Sulfate | 1.0 |
| Distilled Water | 64.0 |
| Methylparaben | 0.025 |
| Propylparaben | 0.015 |
| | 100.040 |

The proportions of liquid petrolatum and white petrolatum in the three petrolatum bases were varied in order to achieve, as nearly as possible, the same consistency among the six test ointments. Fredell's method was used for measuring the relative consistencies of the ointment bases (5). The medicinal agents were incorporated into each of the ointment bases in the following concentrations:

| | |
|---|---|
| Sulfanilamide | 10% |
| Ammoniated Mercury | 5% |
| Salicylic Acid | 5% |
| Chlortetracycline HCl | 3% |
| Iodine and Potassium Iodide | 4% of each |

The first four medicinal agents were incorporated into the ointment bases with no added levigating or intervening agents. The iodine ointments were prepared after the method of Iodine Ointment, N.

[2] Synthetic Japan Wax is produced by International Wax Refining Corp. In a study of 42 waxes and wax-like substances, it was found to be the one most compatible with silicone oils (3).

[3] D C 200 Fluids are silicone oils produced by Dow Corning Corp. Their pharmaceutic properties were reported previously (4).

F. Preparation of the sodium radio-iodide ($I^{131}$) ointments will be discussed later in this paper.

### *In Vitro* Tests of Diffusion

**Microbiological Diffusion Tests.**—This cylinder plate modification of the agar cup plate method has the advantage of allowing testing of all six ointment bases on a single plate, so that each ointment can be compared directly with the other five ointments.

The organism used was *Micrococcus pyogenes* var. *aureus* A.T.C.C. #6538. It was maintained and tested on nutrient agar and was transferred daily into nutrient broth for at least five consecutive daily transfers before the tests were performed.

The ointments were placed in sterile, stainless steel penicylinders (Fischer Scientific #7-907-5). Ten-ml. quantities of melted nutrient agar were poured into sterile 100 mm. x 20 mm. Petri dishes equipped with porcelain covers. When this layer of agar had hardened, a second 10-ml. portion of nutrient agar, which had been seeded with 0.1 ml. of a twenty-four-hour culture of the test organism, was poured over the first agar layer. When this layer, too, had hardened, the ointments, contained in the steel cylinders, were placed on the agar at 60° intervals. The same medicinal agent in six different bases was tested on each plate and a control (the nonmedicated ointment base) was placed in the center of the plate. At least five plates for each medicinal agent were prepared. All plates were incubated at 37° for twenty-four hours, and the extent of diffusion of the antibacterial drugs was determined by measuring the zone of inhibition from the edge of the cylinder to the edge of the inhibition zone.

The above procedure was satisfactory for all the medicated ointments except sulfanilamide ointment for which it was necessary to modify this technique in order to obtain inhibition zones. The diffusion of sulfanilamide from the ointments was determined by the agar cup method rather than the cylinder method, the quantity of the organism suspension was reduced to 0.05 ml. per plate, and the suspension was placed in all 20 ml. of nutrient agar. Zones of partial inhibition were obtained with the emulsion ointments, but not with the anhydrous ointments, by this procedure.

The results of the microbiolgical diffusion tests are shown graphically in Figs. 1–5, where the average sizes in inhibition zones are compared. The legend used in Fig. 1 to designate the ointment bases also applies to the subsequent figures.

When the data were analyzed statistically, the means of the inhibition zones of the various ammoniated mercury ointments were found to be highly significantly different ($P < 0.001$); however, when ammoniated mercury in simple petrolatum ointment was omitted from the analysis, the means of the diffusion of ammoniated mercury from the other five ointments were not significantly different ($P = 0.049$). The means of the diffusion of salicylic acid from ointments were significantly different, even when the ointment showing the lowest diffusion, salicylic acid in simple petrolatum ointment, was not included in the statistical analysis ($0.001 < P < 0.01$). The diffusion of iodine was much greater from the emulsion ointments than from the anhydrous ointments and there was a significant difference in the diffusion from the four anhydrous ointments ($0.001 < P < 0.01$). When all six chlortetracycline ointments were compared, the means of the diffusion zones were found to be highly significantly different ($P < 0.001$); however, when the four anhydrous ointments were compared with each other, the differences in diffusion of chlortetracycline from them were found to be not significant ($P > 0.05$). None of the control ointments produced any inhibition of growth. For a comparison of diffusion, penetration, and absorption from silicone ointment bases and from petrolatum ointment bases, see Table I (page 708).

**Chemical Diffusion Tests.**—In the chemical-agar-tube method, the ointments are superimposed over gels containing a reagent which will react with the medicinal agent to produce colored complexes. The rate and extent of diffusion can be determined by measuring, at frequent intervals, the width of the colored band in the gel. This method was originated by Waud and Ramsay (6) and later used by a number of other investigators (4, 7–11).

The general method used in these tests followed the procedure previously reported by the authors (4). The gels for sulfanilamide, iodine, salicylic acid, and chlortetracycline ointments were prepared by dissolving 4 Gm. of Bacto-Agar (Difco) in each 100 ml. of Ringer's solution and then adding the specific reagent to the warm gel. Ehrlich's reagent was used as the reagent for sulfanilamide determinations and soluble starch was used for the determination of diffusion of iodine from ointments (4). Ferric chloride T. S. was used as the reagent for salicylic acid ointments (0.75 ml. per 100 ml. of Ringer's solution) and for chlortetracycline ointments (0.4 ml. per 100 ml. of Ringer's solution).

Dithizone (diphenylthiocarbazone, Eastman) was used as the reagent for determining diffusion of ammoniated mercury, and the gel was prepared using Pharmagel A®. The preparation of the gel was as follows: An alcoholic solution of dithizone was made by dissolving 100 mg. of dithizone in 20 ml. of alcohol. To 80 ml. of boiling Ringer's solution 20 Gm. of Pharmagel A was added and after this was in solution, and while the solution of the gel was still warm, 4 ml. of the alcoholic dithizone solution was added and the mixture was stirred and poured into test tubes. The ammoniated mercury diffusion zone was shown by a light orange-colored band and at the interface between it and the unreacted gel a more intense pink color was seen.

Three determinations were conducted for each of the thirty ointments. The ointments were placed on the gels and the tubes were stoppered and placed in the dark. At 3-, 6-, 9-, 12-, 18-, 24-, and 36-hour time periods and every eighteen hours thereafter for one week the width of the diffusion bands was measured. The width of the diffusion bands was plotted against time in order to compare the rate of diffusion from the six ointment bases. The extent of diffusion at the twenty-four-hour period is shown in Figs. 1–5; and this time period was chosen for the statistical analyses of results, because it was felt that in clinical use the single application of ointment would not be expected to be effective longer than a twenty-four-hour period.

As seen in Figs. 1 and 5, there was little diffusion of sulfanilamide and of chlortetracycline from the

TABLE I—COMPARISON OF RELATIVE EFFICIENCY OF SILICONE OINTMENT BASES AND PETROLATUM OINTMENT BASES

| Drug | Test Method | Comparison of Results of Diffusion, Penetration, and Absorption: Silicone and Petrolatum Simple Ointments | Silicone and Petrolatum Absorption Ointments | Silicone and Petrolatum Emulsion Ointments |
|---|---|---|---|---|
| Sulfanilamide | Diffusion | | | |
| | Microb. | No inhibition | No inhibition | NSD ($P = 0.190$) |
| | Chem. | Sil. > Pet. | Pet. > Sil. | NSD ($P > 0.5$) |
| | Penetration | | | |
| | Intact | NSD ($P = 0.146$) | NSD ($P = 0.132$) | NSD ($P = 0.011$) |
| | Abraded | NSD ($P = 0.024$) | NSD ($P = 0.119$) | NSD ($P = 0.020$) |
| | Absorption | | | |
| | Intact | No absorption | Pet. > Sil. (see text) | No Absorption |
| | Abraded | Pet. > Sil. ($0.001 < P < 0.01$) | Sil. > Pet. ($0.001 < P < 0.01$) | NSD ($P = 0.247$) |
| Ammoniated Mercury | Diffusion | | | |
| | Microb. | Sil. > Pet. ($P < 0.001$) | NSD ($P > 0.5$) | NSD ($P = 0.145$) |
| | Chem. | NSD ($P = 0.210$) | NSD ($P > 0.5$) | NSD ($P = 0.272$) |
| | Penetration | | | |
| | Intact | NSD ($P = 0.457$) | NSD ($P = 0.144$) | Pet. > Sil. ($0.001 < P < 0.01$) |
| | Abraded | Pet. > Sil. ($0.001 < P < 0.01$) | NSD ($P > 0.5$) | NSD ($P = 0.412$) |
| | Absorption | | | |
| | Intact | NSD ($P = 0.050$) | NSD ($P = 0.382$) | NSD ($P = 0.365$) |
| | Abraded | NSD ($P = 0.060$) | Sil. > Pet. ($0.001 < P < 0.01$) | NSD ($P = 0.165$) |
| Salicylic Acid | Diffusion | | | |
| | Microb. | NSD ($P = 0.027$) | NSD ($P = 0.155$) | NSD ($P = 0.434$) |
| | Chem. | NSD ($P = 0.019$) | NSD ($P = 0.046$) | NSD ($P > 0.5$) |
| | Penetration | | | |
| | Intact | NSD ($P > 0.5$) | NSD ($P = 0.031$) | Pet. > Sil. ($0.001 < P < 0.01$) |
| | Abraded | NSD ($P > 0.5$) | Sil. > Pet. ($P < 0.001$) | NSD ($P > 0.5$) |
| | Absorption | | | |
| | Intact | NSD ($P = 0.103$) | Pet. > Sil. ($0.001 < P < 0.01$) | NSD ($P > 0.5$) |
| | Abraded | NSD ($P = 0.056$) | Sil. > Pet. ($0.001 < P < 0.01$) | NSD ($P = 0.070$) |
| Iodine and Sodium Radiolodide ($I^{131}$) | Diffusion | | | |
| | Microb. | NSD ($P > 0.5$) | Sil. > Pet. ($P < 0.001$) | NSD ($P = 0.301$) |
| | Chem. | NSD ($P = 0.013$) | Sil. > Pet. ($P < 0.001$) | NSD ($P = 0.018$) |
| | Penetration | | | |
| | Intact | NSD ($P > 0.5$) | NSD ($P = 0.108$) | NSD ($P = 0.386$) |
| | Abraded | NSD ($P = 0.043$) | NSD (means are equal) | NSD ($P > 0.5$) |
| | Absorption | | | |
| | Intact | Pet. > Sil. ($0.001 < P < 0.01$) | NSD ($P = 0.205$) | NSD ($P = 0.423$) |
| | Abraded | NSD ($P = 0.113$) | NSD ($P = 0.362$) | NSD ($P = 0.481$) |
| Chlortetracycline HCl | Diffusion | | | |
| | Microb. | NSD ($P = 0.160$) | NSD ($P = 0.037$) | NSD ($P = 0.199$) |
| | Chem. | No difference | Pet. > Sil. | NSD ($P = 0.469$) |
| | Penetration | | | |
| | Intact | NSD ($P = 0.192$) | Sil. > Pet. ($P < 0.001$) | NSD ($P = 0.194$) |
| | Abraded | NSD ($P = 0.369$) | NSD ($P = 0.070$) | NSD ($P = 0.163$) |
| | Absorption | | | |
| | Intact | No absorption of chlortetracycline through intact skin | | |
| | Abraded | Pet. > Sil. ($0.001 < P < 0.01$) | Sil. > Pet. ($0.001 < P < 0.01$) | NSD (means are equal) |

NSD—No significant difference between the silicone ointment and the petrolatum ointment based on $P = 0.010$ or $P < 0.010$ = a significant difference.

Sil. > Pet.—Diffusion, penetration, or absorption was significantly greater from the silicone ointment than from the petrolatum ointment.

Pet. > Sil.—Diffusion, penetration, or absorption was significantly greater from the petrolatum ointment than from the silicone ointment.

two simple ointments and from silicone absorption base. With both of these drugs, the diffusions from the two emulsion ointments did not differ significantly. There was a highly significant difference in the means of the diffusion zones of ammoniated mercury ointments ($P < 0.001$); and although the means of the diffusion of the four anhydrous iodine ointments were of somewhat similar magnitude, they differed highly significantly ($P < 0.001$). When all six salicylic acid ointments were compared with each other, the means were significantly different ($0.001 < P < 0.01$). However, if salicylic acid in simple petrolatum ointment was omitted from the analysis, the differences in diffusion from the other five ointments were not significant.

### *In Vivo* Tests of Penetration and Absorption

**General *In Vivo* Procedure.**—The experimental animals were white female rats of the Sprague-Dawley strain weighing about 175 Gm. Six animals with intact skin and six animals with abraded skin were used to test the penetration and absorption of the medicinal agent from each ointment base. In addition, each ointment base was tested on one animal with intact skin and one animal with abraded skin for each medicinal agent. Thus, there were 72 animals treated with medicated ointments and 12 control animals for each of the five medicinal agents.

The rats were lightly anesthetized with ether, weighed, and the dorsal hair between the shoulder blades and posterior, a distance of about 8 cm., was clipped with a Racine electric clipper. The skin was examined very carefully for reddening or any break in the epidermis. Any animal in which the skin was damaged in any way or in which there was any question about the integrity of the epidermis was used in the abraded-skin study. Clipping the hair completed the preparation of the skin for animals in which the skin was left intact. For those animals used in the abraded-skin study the area which was to be inuncted was abraded with a razor and sandpaper just to the point of reddening and a very slight capillary oozing.

The ointments were confined to the exact test area by use of a dental dam-adhesive tape appliance. The inunction area was exactly 3 x 5 cm. for all ointments except the sodium radio-iodide ($I^{131}$) ointments in which the inunction area was reduced to 2.5 x 2.5 cm. The ointments were applied without pressure for a two-minute period, and then a piece of dental dam was placed over the inuncted area and wrapped around the trunk of the rat. Over this and around the animal was placed a piece of dental dam with openings provided for the animal's legs. This "straight jacket" was taped and cut to fit the rat so that the dorsal surface and sides (except for the head and tail), and the thorax and abdominal areas of the ventral surface were enclosed.

The length of the test period varied with the drug being tested. The objective was to continue the test until the time-period of maximum absorption. For some of the drugs the time element could be determined by consulting the literature. For others it was necessary to perform preliminary absorption-time tests.

At the termination of the test period, the rat was again anesthetized, the dressings were removed, and the skin cleansed of surface ointment. The cleansing process consisted of wiping the excess ointment from the skin, washing the area with a solution of green soap tincture, shaving the skin, washing again with the soapy solution and then rinsing with water. A biopsy of the inuncted skin was then taken for the penetration tests and blood was collected or an organ excised for analysis in order to determine the extent of absorption.

**Penetration and Absorption of Sulfanilamide from Ointments.**—Sulfanilamide ointments were applied in 2-Gm. doses and left in place for five hours. At the end of the five-hour test period, the skin coverings were removed and the skin was cleansed thoroughly. The inuncted skin was then excised and removed for further treatment.

Two one-milliliter blood samples were collected by cardiac puncture, and each was placed in a 125-ml. Erlenmeyer flask which contained 30 ml. of distilled water. The skin biopsy was again washed, and shaved, and triplicate samples, 1 cm. in diameter and each weighing about 100 mg., were obtained. They were blotted on filter paper, weighed, and placed in 10-ml. volumetric flasks for chemical analysis.

The general method devised by Bratton and Marshall (12) was used for sulfanilamide determinations. The sulfanilamide concentration in blood was determined by the procedure as outlined by Hoffman (13), except that the volume of the final solution was increased to 25 ml. Total blood concentrations were determined on animals whose skin was intact and both total and free sulfanilamide concentrations were determined on the blood of animals whose skin had been abraded. The method of Strakosch and Clark (14) was used to determine the concentration of sulfanilamide in skin aliquots except that the final volume was brought to 25 ml. The absorbances of the solutions were determined in a Fisher Nefluro-photometer equipped with a 540-millimicron filter.

The results of the sulfanilamide penetration tests on both intact- and abraded-skin animals are shown in Fig. 1. The means of the penetration of sulfanilamide from the different ointments into intact skin were found to be not significantly different ($P = 0.017$); whereas, in the study of penetration through abraded skin, the penetration from different bases was highly significantly different ($P < 0.001$). The absorption of sulfanilamide from the various ointments through abraded skin is also shown in Fig. 1. The differences in sulfanilamide blood concentrations following application of the six ointments to rats whose skin was abraded were found to be highly significant ($P < 0.001$), and an overall average of 81.7% of the total blood sulfanilamide was present as free sulfanilamide. When the results of the determinations on the blood of intact-skin animals which were treated with sulfanilamide ointments were compared with the results from the rats treated with control ointments, it was found that sulfanilamide in petrolatum absorption base was the only ointment which gave a significant sulfanilamide blood concentration ($0.001 < P < 0.01$) after application to intact skin.

**Penetration and Absorption of Ammoniated Mercury from Ointments.**—In tests of percutaneous absorption of mercury, Laug, *et al.* (15) found that a

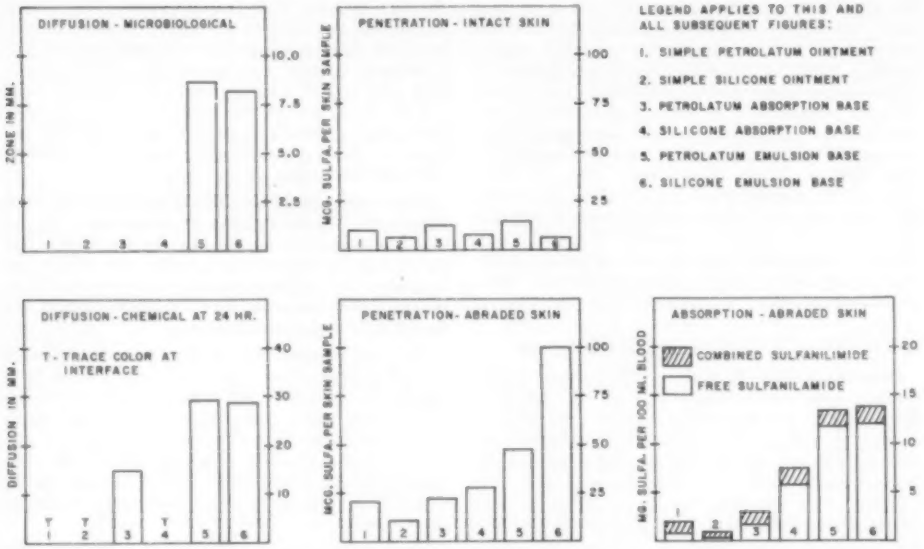


Fig. 1.—A comparison of diffusion, penetration, and absorption tests of sulfanilamide from silicone and petrolatum ointments.

large proportion of the mercury absorbed was concentrated in the kidneys. In this investigation many of the procedures are similar to the ones used by Laug and his associates in their investigations of the absorption of mercury and mercury salts (15–18).

The ammoniated mercury ointments in 2-Gm. doses were applied and allowed to remain in place for a twenty-four-hour period. The total inuncted skin area was thoroughly cleansed, weighed, and placed in a 250-ml. flat-bottom Pyrex boiling flask for analysis.

The method used for the analysis of mercury in the skin and kidneys was a modification of the dithizone method developed by Laug and Nelson (19). The two kidneys from each animal were placed in a 250-ml. flat-bottom boiling flask. Two milliliters of sulfuric acid, reagent grade; and 0.6 ml. of nitric acid, reagent grade; were added and

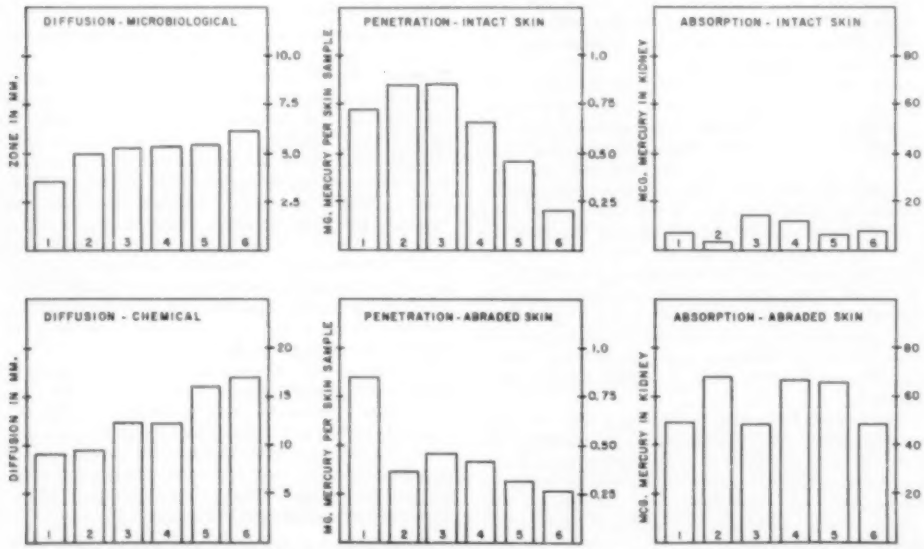


Fig. 2.—A comparison of diffusion, penetration, and absorption tests of ammoniated mercury from silicone and petrolatum ointments.

the flask was connected to an Allihn condenser. All Pyrex and ground-glass connected apparatus was used. The sample was heated very slowly at first and then more strongly until all of the tissue was taken into solution and the solution became straw-colored (about two hours). Usually a small additional quantity of nitric acid was necessary to oxidize completely the animal tissue. The solution was then cooled, placed in a 50-ml. volumetric flask, and distilled water was added to volume.

A 50-ml. quantity of 0.25 *N* HCl[4] was measured into a 250-ml. separatory funnel and to this was added a 10-ml. aliquot of the kidney solution. The solutions were mixed, 5 ml. of 20% hydroxylamine hydrochloride was added, and the funnel shaken. Then 25 ml. of dithizone solution was added, the funnel shaken 60 times, and the concentration of mercury dithizonate determined by comparing the absorbance (at 490 millimicrons) of the chloroformic phase with the standard curve.

The procedure for the skin samples was very similar. The skin was digested with 5 ml. of sulfuric acid and 2 ml. of nitric acid. The solution was then made to volume in a 100-ml. volumetric flask and the absorbance of a 1-ml. or 2-ml. aliquot was determined.

The penetration and absorption of ammoniated mercury from each of the six ointment bases are compared graphically in Fig. 2. In the animals with intact skin, the means for the penetration of ammoniated mercury showed a highly significant difference ($P < 0.001$); but when the four anhydrous bases were compared, the means of the penetration from them showed no significant differences. In penetration studies of animals with abraded skin, the penetration from simple petrolatum ointment was much higher than from the other five ointment bases and the differences in the penetration from the other five ointments were not significantly different ($P = 0.049$) from each other. Through intact skin, absorption was found to differ highly significantly from the six ointment bases ($P < 0.001$); whereas, through abraded skin differences in the absorption of mercury from the six ointments was insignificant ($P = 0.050$).

The color of the mercury-dithizone complex was not stable for more than a few minutes. Therefore, the absorbances of the solutions were determined immediately after extraction. In addition, the kidneys of control animals contained some substance which reacted with dithizone to give a colored complex that could not be differentiated, in spite of exhaustive experimentation (20) from the mercury-dithizone complex. Since no explanation of the high control value phenomenon could be found, the average value for the kidneys of control rats (3.8 mcg.) was subtracted from the value found for each of the treated rats.

**Penetration and Absorption of Salicylic Acid from Ointments.**—Rats were treated with 2-Gm. doses of each of the salicylic acid ointments and the ointments were kept in place for a three-and-one-quarter-hour period. At the end of the test period, the ointment was removed, blood was collected by cardiac puncture, and the inuncted skin was removed.

With few modifications, the salicylate procedure devised by Brodie, *et al.* (21), was used for determining salicylic acid in rat plasma. The volume of water used in the final extraction was increased to 25 ml. and 0.5 ml. of ferric nitrate (1% in 1% nitric acid) was used as the reagent. The absorbance of the violet iron-salicylate complex was determined at 525 mμ and compared with a standard curve.

An adaptation of the method used for analysis of salicylic acid in plasma was used for the determination of salicylic acid in skin samples. Skin aliquots 1.8 cm. in diameter were placed in 50-ml. Pyrex glass-stoppered bottles and 0.5 ml. of 6 *N* hydrochloric acid and 2 ml. of distilled water were added. The bottles were placed in a boiling-water bath for thirty minutes, shaken vigorously in order to break up the tissue, and then 30 ml. of ethylene dichloride was added, and the assay conducted as for plasma.

The penetration and absorption of salicylic acid from each of the ointment bases is shown in Fig. 3. Through both intact and abraded skin the differences in the penetration of salicylic acid from the six ointments were found to be highly significant ($P < 0.001$). In the intact-skin study, when salicylic acid in petrolatum absorption base was omitted from the statistical analysis, the differences in the penetration of salicylic acid from the other five ointments were found to be insignificant ($P = 0.048$); however, the difference between the ointment showing the highest penetration and the one with the lowest in this series of five ointments, was highly significant ($P < 0.001$). In the abraded-skin study, when salicylic acid in petrolatum absorption base was omitted from the statistical analysis, the differences in the penetration of salicylic acid from the other five ointments were found to be insignificant. In comparing absorption of salicylic acid from the six ointments through both intact skin and abraded skin, the differences in the means of the plasma concentrations following application of the salicylic acid ointments were found to be highly significant ($P < 0.001$).

**Penetration and Absorption of Sodium Radio-Iodide ($I^{131}$) from Ointments.**—The penetration and absorption of sodium radio-iodide ($I^{131}$) from the six ointment bases was studied by a method similar to the one used by Cyr, *et al.* (22), and Skauen, *et al.* (23), in which the radioactivity of the thyroid gland is determined as a measure of $I^{131}$ absorption. The radioactivity of aliquots of the $I^{131}$ ointment-treated skin was determined as a measure of the penetration of the sodium iodide.

The ointments were prepared immediately before use and each rat was treated with a 200-mg. dose of ointment which contained:

| | |
|---|---|
| Sodium Radio-Iodide ($I^{131}$), | 10 microcuries |
| Sodium Iodide, | 20 mg. |
| Ointment Base, | 180 mg. |

Concurrently with the preparation of the $I^{131}$ ointments a standard solution was prepared so that a 0.1-ml. aliquot of the solution contained $5 \times 10^{-4}$ microcuries or $1/(2 \times 10^6)$ of the 10-microcurie $I^{131}$ dose. This solution was used in comparing the activity of the thyroid and skin tissues of treated

[4] The reagents used were similar to those described by Laug and Nelson (19): HCl—glass distilled and then diluted to 0.25 *N*. Hydroxylamine HCl, 20% w/v—it was found unnecessary to extract the solution with dithizone. Dithizone solution—4 mg. per liter in glass distilled chloroform.

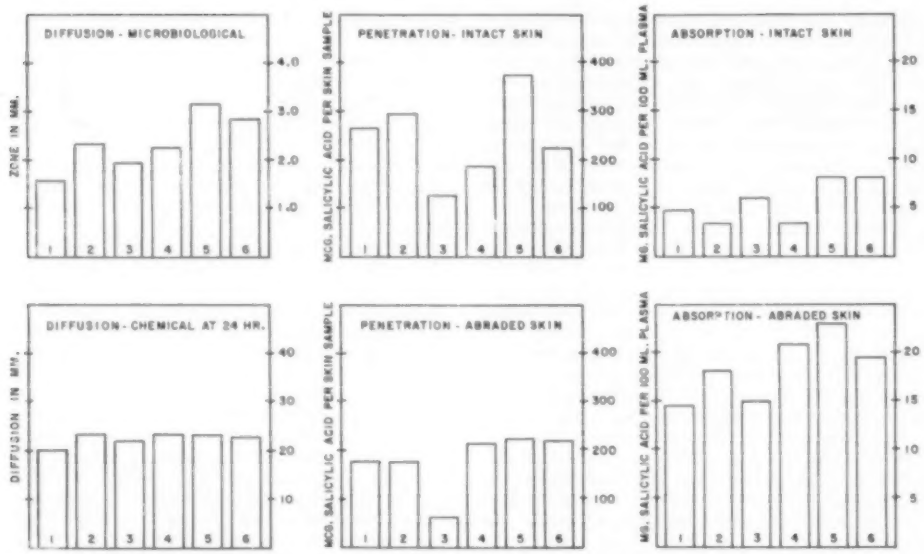
DIFFUSION - MICROBIOLOGICAL
ZONE IN MM.
PENETRATION - INTACT SKIN
MCG. SALICYLIC ACID PER SKIN SAMPLE
ABSORPTION - INTACT SKIN
MG. SALICYLIC ACID PER 100 ML. PLASMA
DIFFUSION - CHEMICAL AT 24 HR.
DIFFUSION IN MM.
PENETRATION - ABRADED SKIN
ABSORPTION - ABRADED SKIN

Fig. 3.—A comparison of diffusion, penetration, and absorption tests of salicylic acid from silicone and petrolatum ointments.

rats with the activity of the total $I^{131}$ which had been applied.

The ointments were left in place for a thirty-hour period and at the end of that time a biopsy of the washed, inuncted skin 1.25 cm. in diameter was carefully removed and placed in a 4-dram, screw-capped vial which contained 2 ml. of 10% sodium hydroxide solution. The thyroid gland, the larynx, and the first three cartilaginous rings of the trachea below the isthmus were excised and placed in a 1-dram, screw-capped vial containing 1 ml. of 10% sodium hydroxide solution.

After the samples had been digested in the sodium hydroxide solution for twenty-four hours, the vials were shaken vigorously to complete the dispersion of the tissue throughout the liquid. A 0.1-ml. aliquot of the tissue suspension was pipetted (using a pro-pipet) onto a copper planchet 2.5 cm. in

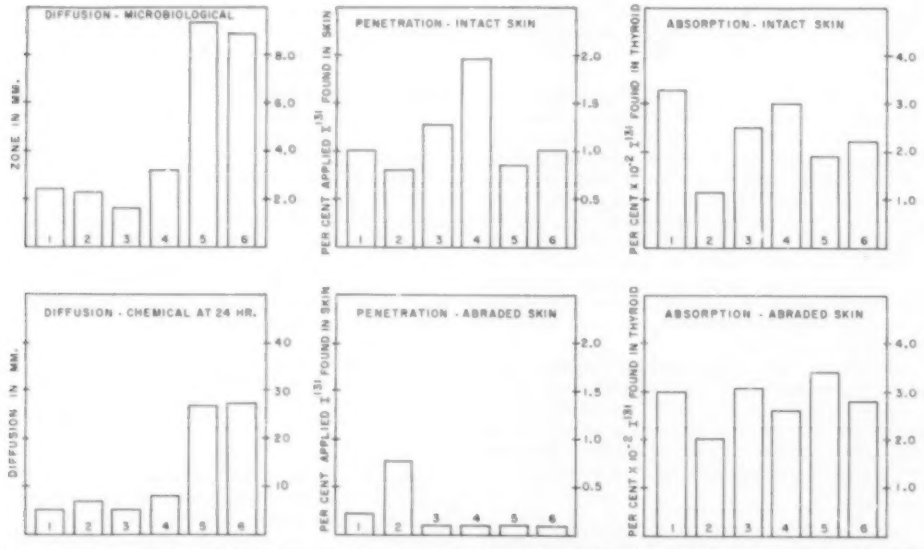
DIFFUSION - MICROBIOLOGICAL
ZONE IN MM.
PENETRATION - INTACT SKIN
PER CENT APPLIED I131 FOUND IN SKIN
ABSORPTION - INTACT SKIN
PER CENT X 10-2 I131 FOUND IN THYROID
DIFFUSION - CHEMICAL AT 24 HR.
DIFFUSION IN MM.
PENETRATION - ABRADED SKIN
ABSORPTION - ABRADED SKIN

Fig. 4.—A comparison of diffusion, penetration, and absorption tests of iodine and sodium radio-iodide ($I^{131}$) from silicone and petrolatum ointments.

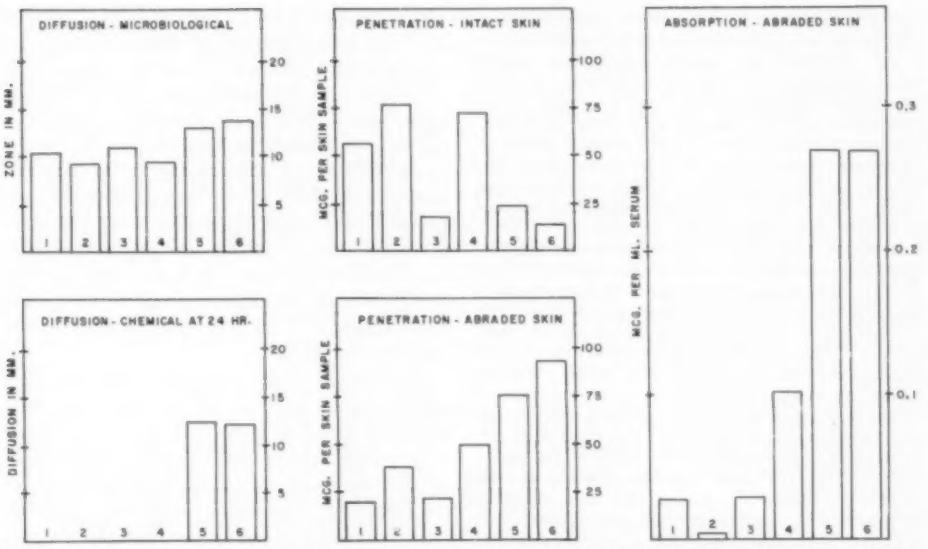


Fig. 5.—A comparison of diffusion, penetration, and absorption tests of chlortetracycline hydrochloride from silicone and petrolatum ointments.

diameter. The suspension was dried under a Penetray infrared heat lamp and the planchet was placed in a desiccator. Silicone grease, DC Compound 4 (Dow Corning), was spread in a very fine ribbon around the perimeter of the planchet to prevent the suspension from spreading over the edge.

In preparing standards, 0.1 ml. of 10% sodium hydroxide solution was placed on the planchet, the solution was evaporated, and then 0.1 ml. of the standard solution was pipetted onto the planchet, and the planchet was dried under the heat lamp.

The radioactivity of the samples was counted in a Tracerlab, model SC-16 windowless flow counter with which a Sidl model 183 scaling unit was used.

The percentage of the $I^{131}$ dose which was found in the skin and in the thyroid following application of each of the $I^{131}$-containing ointments is shown in Fig. 4. In both intact- and abraded-skin studies, when the ointment showing the greatest penetration was omitted from the statistical analysis, the penetrations from the other five ointment bases were not significantly different. The differences in the absorption of $I^{131}$ through intact skin from the six ointment bases were found to be highly significant ($P < 0.001$); however, through abraded skin the differences in absorption of iodide ($I^{131}$) were found to be statistically insignificant ($P > 0.05$).

**Penetration and Absorption of Chlortetracycline from Ointments.**—The cylinder-plate method of microbiological assay (24, 25) was used to determine the concentration of chlortetracycline in serum and skin following application of the six chlortetracycline ointments.

One-gram doses of ointment were applied and left in place for a five-hour test period after which the skin was thoroughly cleansed and wiped with a pledget of cotton which had been moistened in alcohol 70% W/W. All equipment and solutions used were sterile. A skin aliquot, 1.5 cm. in diameter, was placed in a tissue grinder (Arthur H. Thomas Company, No. 4288-B) connected to a small motor. Two milliliters of phosphate buffer, pH 4.5 (25), was added to the pieces of skin in the grinder and the tissue was ground until a homogeneous suspension was attained. The tissue suspension was poured into a 4-dram vial, the extraction was repeated with three 1-ml. portions of phosphate buffer and these extracts were added to the skin homogenate in the vial.

Blood was collected by cardiac puncture and the serum was placed in 1-dram vials. Both skin homogenates and serum were stored in a deep freeze at −20° until a convenient time for analysis.

The test organism was *Bacillus cereus* var. *mycoides* and the spore suspension was prepared according to the procedure outlined by Grove and Randall (25). Difco media for both base and seed layers were used as suggested in the test method (24). It was found that the organism grew better at a pH of 6.6 (the pH of the Difco medium) than when the pH was adjusted as specified in the assay to 5.6.

Standard curve determinations for both serum and skin were conducted. Serum was collected from untreated rats, and standard solutions of chlortetracycline for the serum curve were prepared using one part of this normal serum to two parts of a mixture of a standard chlortetracycline hydrochloride solution and phosphate buffer pH 4.5 such that the solutions ranged in concentration from 0.005 to 0.24 mcg. per ml. In the preparation of solutions used in the determination of a standard curve for assay of skin samples, a piece of untreated rat skin of the same size as the skin samples of the treated rats was ground in the tissue grinder and suspended in 5 ml. of phosphate buffer. One milliliter of the skin

homogenate was added to a 9-ml. combination of chlortetracycline standard solution plus phosphate buffer. For the skin standard curve, standards were prepared to contain 0.01 to 4.5 mcg. of chlortetracycline hydrochloride per ml. of solution. The plates were prepared according to Grove and Randall's description (25) and the resulting inhibition zones were plotted against the log of the chlortetracycline concentrations to produce a straight-line curve. The reader is referred to Grove and Randall for details on determining the standard curve (25).

In the determination of the concentration of chlortetracycline in the skin of treated animals, 1 ml. of the skin homogenate was mixed with 9 ml. of phosphate buffer in a 4-dram screw-capped vial. For determination of serum concentration of the antibiotic, a 1-ml. sample of the unknown serum was mixed with 2 ml. of phosphate buffer in a 1-dram screw-capped vial.

Plates were prepared and analyses carried out using seven cylinders per plate (six cylinders at 60° intervals and a seventh in the center of the plate) and one plate for each unknown solution or skin suspension. Two cylinders at 180° to each other were filled with standard solution and the other five cylinders were filled with the unknown solution. The concentration of the unknown sample was determined by comparing the inhibition zone of the unknown with standards on the same plate and the standard curve (25).

The penetration and absorption of chlortetracycline from each of the ointments is shown in Fig. 5. The differences in the penetration of chlortetracycline from the different ointments were highly significant in both intact-skin studies and abraded-skin studies ($P < 0.001$). All the skin samples of rats treated with control ointments showed inhibition zones of the approximate size of the outside diameter of the steel penicylinders, and this same inhibition zone was shown by cylinders containing only the phosphate buffer. The fact that *Bacillus cereus* does not grow well at the pH of the phosphate buffer is an explanation for the phenomenon. In the serum controls, which contained two-thirds phosphate buffer and one-third serum of the control animals, no growth inhibition occurred.

Chlortetracycline was not absorbed through intact skin as shown by complete lack of inhibition zone with the serum of any of the 36 intact-skin animals which were treated with chlortetracycline ointments. Through abraded skin chlortetracycline was absorbed by only one of the six animals treated with chlortetracycline in simple silicone ointment.

## DISCUSSION AND CONCLUSIONS

Examination of Figs. 1–5 enables a comparison of the results obtained by *in vitro* tests of diffusion and *in vivo* tests of penetration and absorption.

The results of the two *in vitro* procedures for evaluating the diffusion of the medicinal agent from the ointment bases correlated fairly well with each other for three of the medicinal agents: sulfanilamide, iodine, and chlortetracycline hydrochloride; and correlated slightly for the other two: ammoniated mercury and salicylic acid.

The *in vitro* diffusion data correlated with *in vivo* data from the study of penetration into intact skin with only one of the five drugs—salicylic acid, and this correlation was very slight. In a comparison of *in vitro* diffusion data and *in vivo* data on penetration into abraded skin, there was a fair measure of agreement in the results of tests of sulfanilamide and chlortetracycline ointments, a very slight correlation in the results of *in vitro* and *in vivo* salicylic acid ointment evaluations, and no correlation in *in vitro* diffusion and *in vivo* penetration results for iodine and sodium radio-iodide ($I^{131}$) ointments and for ammoniated mercury ointments.

For the four medicaments which were absorbed through intact skin, the *in vivo* absorption data were not found to agree with *in vitro* diffusion data except for the salicylic acid ointments in which there was some agreement in the results of the two tests. Through abraded skin, however, the relative *in vivo* absorption from the six ointment bases agreed fairly well with the *in vitro* results for three of the five groups of medicated ointments: sulfanilamide, salicyclic acid, and chlortetracycline; and did not agree for the two others, although there was a very slight correlation between the absorption test and the microbiological diffusion test for ammoniated mercury ointments.

It could not be shown that either the silicone-based ointments or the petrolatum-based ointments were universally the more efficient. In Table I the two simple ointments, the two absorption ointments, and the two emulsion ointments were compared statistically with each other. Eighty-two silicone ointment-petrolatum ointment comparisons were made and of these, 62 comparisons showed no significant difference in the diffusion, penetration, or absorption of drugs from the silicone ointments or from the petrolatum ointments. In 10 tests silicone ointments were the more efficient vehicles, and in 10 other tests the petrolatum ointments were the more efficient.

The results of penetration and absorption studies in this investigation serve well as a basis for evaluation of the questionable theory of the "therapeutic classification" of ointments. The simple ointments used in this study would fall into the epidermatic classification; the absorption bases, because of their lanolin content, would be considered endodermatic; and the emulsion bases would be classified as diadermatic. With sulfanilamide and chlortetracycline ointments it is seen that the ointments classed diadermatic do permit or promote, in abraded-skin animals,

greater skin penetration and systemic absorption than do the ointments coming under the other two classifications. The differences between the so-called epidermatic and endodermatic ointments are slight for these drugs. Relative sulfanilamide and chlortetracycline penetration into intact skin is not what would be expected from the ointment therapeutic classification. In relative penetration and absorption of ammoniated mercury from the three types of ointments, it is seen that the experimental data do not support the theoretical therapeutic classification. For iodide ointments the penetration into intact skin is greater from the absorption bases than from the simple and emulsion ointments. This fact would be expected from a theoretical standpoint if it were postulated that the drug, when it is incorporated in an emulsion base, moves through the skin into systemic circulation rather than remaining in skin tissue in a high concentration. The absorption data through both intact and abraded skin, though, do not support the therapeutic classification theory. The relative absorption of salicylic acid from the ointment bases is what might be expected from the therapeutic classification; but the penetration, which according to the therapeutic classification should be highest from the absorption ointments, is low from these bases. Thus, it is seen that the theory of therapeutic classification of ointments has less than 35 per cent support by the experimental data of this investigation. It is seen, too, in examining the data that penetration and absorption are not wholly dependent upon the vehicle, that relative penetration and absorption of different drugs from a series of ointment bases are not in the same order for all medicinal agents, and that drugs vary greatly in their ability to penetrate through normal intact skin.

The present trend seems to be toward use of emulsion ointments or creams whenever the stability of the active constituent allows use of such a vehicle. For local treatment in dermatologic conditions, the clinician usually desires a high concentration of the therapeutic agent in the skin and low or no systemic absorption of the drug. When the skin was intact, the drug concentration in the skin was usually higher following application of anhydrous ointments than following application of emulsion ointments. In animals with abraded skin, sulfanilamide and chlortetracycline concentrations in the skin were higher following application of these drugs in the emulsion ointments than in the anhydrous bases; however, with iodine and ammoniated mercury ointments, the emulsion ointments gave low skin-tissue concentrations. With these latter two ointments, the test period was long, and it may be that the tissue fluid, which was lost as a result of skin abrasion, washed the medication out of the skin. This may indicate that unless dressings over an abraded area are changed frequently, higher drug concentrations in the skin will be achieved with anhydrous ointments than with emulsion bases. The high systemic absorption of sulfanilamide, ammoniated mercury, and salicylic acid following application of these ointments to abraded skin emphasizes the possible systemic toxicity in topical application of drugs to abraded surfaces.

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# A Cylinder-Plate Method for the Quantitative Determination of Diffusion of Antibacterial Drugs from Ointments*

By ELMER M. PLEIN and JOY BICKMORE PLEIN

**Plotting the squares of the diameters of the inhibition zones produced by varying concentrations of antibacterial drugs against the logarithms of the concentrations resulted in straight line curves for mercury bichloride, chlortetracycline hydrochloride, oleandomycin phosphate, salicylic acid, and sodium sulfacetamide. A cylinder-plate method for quantitative determination of diffusion of antibacterial drugs from ointments by reference to the standard curves is presented. The results of simple cylinder-plate-inhibition zone diffusion tests in six different ointment bases are compared with the quantitative data obtained in testing diffusion from the six ointments by the cylinder-plate standard curve method.**

THE MOST WIDELY USED method of ointment base evaluation is by means of the agar plate or agar cup plate procedures or modifications of these methods. Ruehle and Brewer (1) in Circular 198 of the U. S. Department of Agriculture described these two procedures for determining the diffusion of antibacterial drugs from ointments, and although there have been numerous modifications of their methods, the procedures have not changed essentially.

In the assay of a number of different antibiotics, penicylinders are placed on agar plates which have been seeded with a microorganism susceptible to the antibiotic. The penicylinders are then filled with solutions containing varying concentrations of the antibiotic and the resulting inhibition zones are plotted against the logarithms of the concentrations in constructing a standard curve. The potency of unknown antibiotic solutions is then determined by reference to the standard curve (2, 3). Since this logarithmic concentration-inhibition zone relationship is true for antibiotic solutions, it was felt that this function or other mathematical relationships might also be valid for other antibacterial drugs.

This study was undertaken in order to determine whether the inhibition zones produced by varying concentrations of several antibacterial drugs were functions of the drug concentrations, to construct standard curves from the data if this relationship were found, and to use these standard curves in obtaining quantitative data on diffusion of antibacterial drugs from ointments.

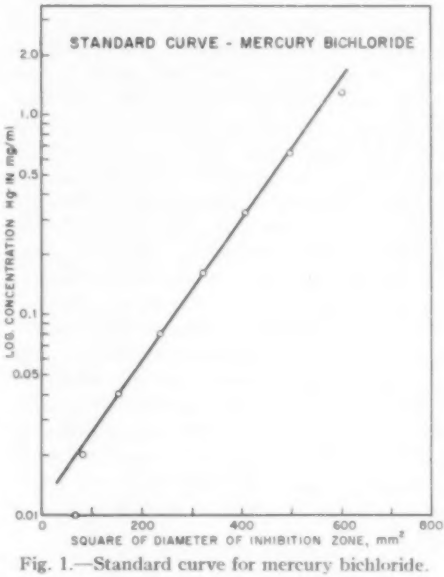


Fig. 1.—Standard curve for mercury bichloride.

## EXPERIMENTAL

Ammoniated mercury, chlortetracycline hydrochloride, oleandomycin phosphate, salicylic acid, and sodium sulfacetamide were selected for this study.

*Micrococcus pyogenes* var. *aureus* (*Staph. aureus*), A. T. C. C. #6538, was used as the test organism for all five drugs and in addition, *B. cereus*, A. T. C. C. #9634, was used in the chlortetracycline study. The *Staph. aureus* was maintained and tested on nutrient agar prepared by rehydrating Difco Nutrient Agar 0001-01. The organism was grown for testing in nutrient broth containing 5.0 Gm. of peptone and 3.0 Gm. of beef extract per 1000 ml. of distilled water. The usual bacteriological media inhibit sulfonamide action (4, 5). Therefore, for sodium sulfacetamide standard curve and ointments the medium of Strauss, *et al.* (4), was used to

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Presented to the Scientific Section, A. PH. A., New York meeting, May 1957.
The silicone oils were supplied by Dow Corning Corp., the chlortetracycline hydrochloride by Lederle Laboratories, Division of American Cyanamid Co., and the oleandomycin phosphate by Pfizer Laboratories, Division of Charles Pfizer and Co.

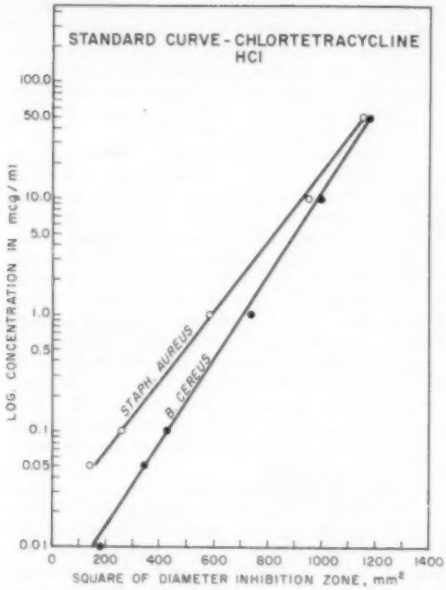


Fig. 2.—Standard curves for chlortetracycline hydrochloride.

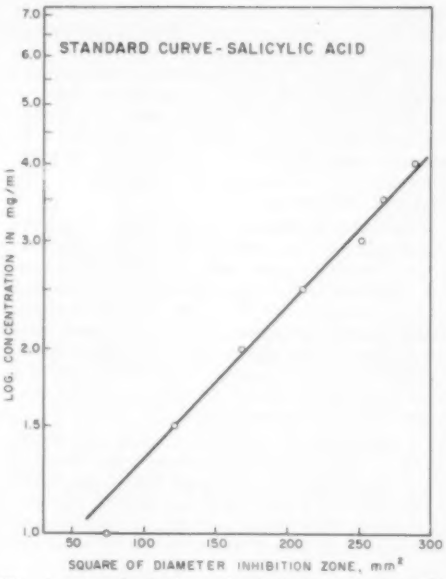


Fig. 4.—Standard Curve for Salicylic Acid in 20% Alcohol.

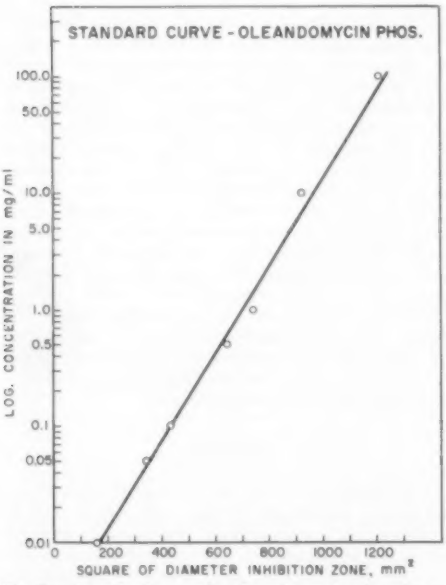


Fig. 3.—Standard curve for oleandomycin phosphate.

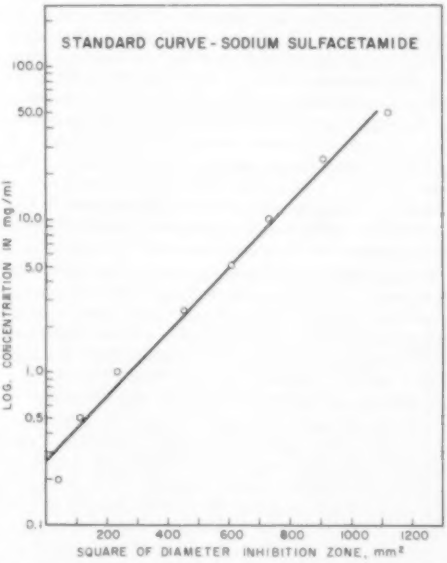


Fig. 5.—Standard Curve for Sodium Sulfacetamide.

grow the *Staph. aureus* and a solid medium for the agar plates was prepared by the addition of 15.0 Gm. of Bacto-agar (Difco) per liter of gel to the Strauss, *et al.*, formula.

The *B. cereus* was maintained on rehydrated Difco penassay seed agar 0263-02 and a spore suspension of the organism was prepared as described by Grove and Randall (2).

**Preparation of Test Solutions.**—Sterile technic and sterile diluent were used in preparing the solutions. The antibacterial concentrations used may be seen by referring to the points in Figs. 1 to 5.

Distilled water was used as the diluent for oleandomycin phosphate and for sodium sulfacetamide solutions. Phosphate buffer pH 4.5 (2) was used as the diluent for chlortetracycline solutions and alcohol 20% v/v was used as the diluent for salicylic acid. An aqueous solution of mercury bichloride was used in determining a standard curve for mercury, and diffusion of ammoniated mercury ointments was determined by reference to this curve (see Discussion).

**Preparation of Plates.**—*Staph. aureus* organisms were transferred into appropriate nutrient broth for at least five consecutive daily transfers before use. Ten milliliters of the appropriate melted nutrient agar was poured into 20 x 100 mm. petri dishes equipped with porcelain covers. The agar layer was allowed to cool and harden, and then a 10-ml. quantity of nutrient agar seeded with 0.1 ml. of a twenty-four-hour culture of *Staph. aureus* was poured over the first agar layer.

For the testing of chlortetracycline solutions and ointments with *B. cereus*, the plates were prepared as described by Grove and Randall (2) for chlortetracycline solutions. Difco penassay medium was used for these determinations, and 0.1 ml. of the spore suspension of *B. cereus* was mixed with the second layer.

**Standard Curve Determinations.**—When the seeded layer had hardened, porcelain penicylinders (Fischer Scientific) were placed on the agar surface. With all drugs, except chlortetracycline, six cylinders per plate were placed on the agar at 60° intervals with the center of the cylinder at about a 3.5-cm. radius. A seventh cylinder was placed in the center of the plates which were used for low concentration solutions in order to determine that the diluent itself was not bacteriostatic. A solution midpoint in the range of concentrations of those being tested was selected as the reference solution and was placed in three alternating cylinders on each plate. The other three cylinders were filled with a test solution of another potency, and the seventh cylinder in the center of the plate was filled with the solution diluent. Three plates were prepared for each concentration.

The inhibition zones obtained in testing chlortetracycline ointments were large, and therefore, only four cylinders were placed on each plate used in chlortetracycline determinations. For the standard curve, alternate cylinders were filled with the reference solution and with another test solution, and four plates were prepared for each concentration.

The plates were incubated at 34° for seventeen hours (*B. cereus* at 30°) and the diameters of the zones of inhibition were read by placing the plate over an AO Spencer colony counter. These inhibition zones were corrected (2, 3) by referring to the reference solution, and a standard curve was constructed by plotting the squares of the diameters of the inhibition zones (on the arithmetic scale) against the logarithms of the concentrations of the antibacterial drug (on the logarithmic scale) on semilogarithmic paper. The standard curve was drawn in such a way that the curve invariably went through the point representing the reference solution.

**Diffusion of Drugs from Ointments.**—Six ointment bases: simple petrolatum ointment, simple silicone ointment, petrolatum absorption base, silicone absorption base, petrolatum emulsion base, and silicone emulsion base were prepared (6). Each of the medicinal agents was incorporated in each of these six ointment bases in the following concentrations: ammoniated mercury 5%, chlortetracycline hydrochloride 3%, oleandomycin phosphate 3%, salicylic acid 5%, and sodium sulfacetamide 10%. Thus, 30 ointments were tested.

The plates were prepared just as those for the standard curves. Three cylinders were placed at 120° intervals on each plate and these cylinders were filled with the same reference solution which was used as the reference solution for the standard curve. For each plate three cylinders were filled with the test ointment and these cylinders were placed on the plates between the cylinders containing the reference solution. Three plates were prepared for each of the six ointment bases in testing the diffusion of a particular medicinal agent. Two cylinders containing a chlortetracycline ointment and two cylinders containing chlortetracycline reference solution were used on each plate for testing chlortetracycline ointments, and four plates were prepared for each ointment.

The plates were incubated and the inhibition zones read as with the standard curve determinations. The mean of the inhibition zones of the 54 reference solution determinations was calculated and the mean of the three reference solution determinations for each plate was also calculated. The mean inhibition zone for the three ointments on each plate was corrected to what it would have been had the mean inhibition zone of the reference solution on that plate been equal to the mean inhibition zone of the 54 determinations of the reference solution. The mean inhibition zone of the reference solution for all the ointment determinations usually varied slightly from the mean of the reference solutions in the standard curve determination. Therefore, a line was drawn through the point representing the reference solution of the ointment determinations and parallel to the standard curve. The concentrations of active medicinal agent which had diffused from the ointments could then be read directly from the standard curve (corrected for this particular group of data) by squaring the corrected diameters of the ointment inhibition zones and reading across the graph to the concentration.

Standard curves for chlortetracycline were conducted with both *Staph. aureus* and *B. cereus*, and since the inhibition zones were more distinct in the *B. cereus* determination, this organism was used for the chlortetracycline ointment tests.

## RESULTS

The results of the standard curve determinations are shown graphically in Figs. 1–5. As is seen in these figures, a good straight line curve is obtained when the logarithms of the concentrations of the antibacterial drugs are plotted against the squares of the diameters of the zones of inhibition. This relationship was found to hold true whether concentrations extending over only one cycle were needed for assay of ointments (salicylic acid ointments), or whether a concentration range of five cycles was needed (oleandomycin ointments).

The results of quantitative determination of the diffusion of the five drugs (in mg./ml. obtained from standard curves) from each of the six ointment

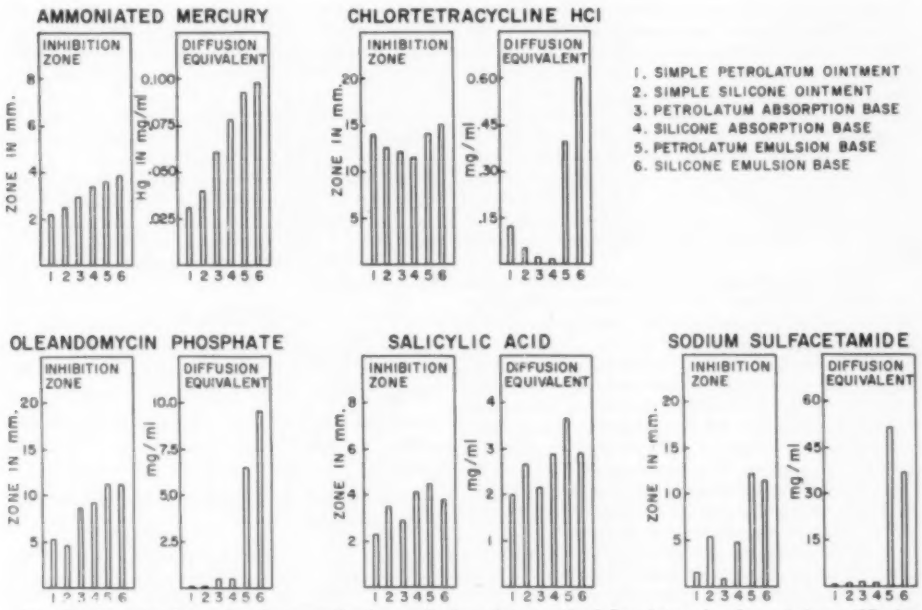


Fig. 6.—Results shown by the quantitative cylinder plate method for determination of drug diffusion from ointments compared with results shown by inhibition zone measurement alone. "Diffusion equivalent" refers to mg./ml. on the standard curve. Inhibition zones reported are from edge of cylinder to edge of zone of inhibition.

bases is shown in Fig. 6 (right half of each section). For purpose of comparison the uncorrected inhibition zones (in mm. from edge of cylinder to edge of inhibition zone) from the same data are also shown in Fig. 6 (left half of each section). The importance of the determination of logarithmic concentration-inhibition zone relationships in evaluation of diffusion of drugs from topical vehicles is apparent by comparing the inhibition zones produced by these 30 ointments with the actual quantity of medicinal agent diffused from the ointments. It may be seen that with salicylic acid and ammoniated mercury the inhibition zones as determined by the cylinder-plate method gave an indication of the relative diffusion from the six ointment bases. However, with ammoniated mercury, the differences in inhibition zones were not proportional to the differences in ammoniated mercury actually diffused. With the other three medicinal agents, the results obtained by the cylinder-plate method and the actual determination of diffusion by reference to a standard curve are not even comparable. For example, the inhibition zone of oleandomycin in silicone emulsion base was only slightly greater than twice the zone produced by the drug in simple petrolatum ointment, but the amount of oleandomycin diffused from silicone emulsion base was 345 times the quantity diffused from the simple petrolatum ointment. Also, the inhibition zones produced by the different chlortetracycline ointments varied only from 11.6 mm. to 14.8 mm., whereas the quantity of chlortetracycline diffused from these ointments varied from 17.5 to 600 mcg./ml. Other comparisons of the data of this investigation emphasize that only by use of a standard curve will inhibition zones show a valid evaluation of drug diffusion.

## DISCUSSION

The usual method for the assay of antibiotics (2, 3) employs the diameters of the zones of inhibition instead of the squares of the diameters. This procedure yields good results since the range of the concentrations is relatively small. In this study, when the diameters of the zones of inhibition were plotted against the logarithms of the concentrations, a good straight line curve did not result if the concentrations were extended through a wide range. With salicylic acid it was necessary to extend the concentrations through only one cycle in order to produce inhibition zones both greater than and less than those produced by the salicylic acid ointments, and a fairly straight line was obtained when the diameters of the inhibition zones were plotted against the logarithms of the salicylic acid concentrations. With the other four drugs, whose concentrations extended over three to five logarithmic cycles, the standard curves were decidedly curved when the data were plotted in this manner (using diameters rather than diameters squared).

It should be pointed out that use of a reference solution on each plate is critically necessary for accurate determinations since certain important factors, principally temperature variables, cannot be absolutely controlled. The importance of the reference solution to the accuracy of agar plate methods

is evidenced by examination of the data for oleandomycin diffusion from petrolatum emulsion base and from silicone emulsion base. While there was no difference in the uncorrected inhibition zones produced by the two ointments, after correction the inhibition zone of oleandomycin in silicone emulsion base was found to be 0.75 mm. greater than that of the drug in petrolatum emulsion base. Since these zones were large (about 31 mm.), this small difference (0.75 mm.) accounted for a difference in antibiotic diffusion of 3.1 mg./ml., or an increase of about 50% (compare standard curve with the data in Fig. 6).

In order to introduce practical problems involving the diffusion of insoluble or relatively i..soluble drugs from ointments, ammoniated mercury and salicylic acid were included in this study. Inasmuch as ammoniated mercury is insoluble in water and no satisfactory agent was found to solubilize the drug, mercury bichloride was selected for the determination of the standard curve, and concentrations were calculated on a basis of mercury content. It was felt that this liberty in using mercury bichloride to evaluate diffusion of ammoniated mercury was justified because it is the mercury ion which is responsible for the activity of ammoniated mercury.

Salicylic acid is not sufficiently soluble in water to produce solutions of the strength necessary to construct a standard curve which will extend over the range of the inhibition zones produced by the salicylic acid ointments. Twenty per cent alcohol v/v was selected as the solvent for this drug after it was determined that *Staph. aureus* developed colonies beneath solutions containing alcohol in 40% v/v concentrations or less. Since it was thought that 20% alcohol might potentiate the activity of salicylic acid, inhibition zones produced by an aqueous solution of salicylic acid, 2 mg./ml., were compared statistically with the zones produced by the acid in 20% alcohol. The alcoholic solutions produced slightly larger zones than did the aqueous solutions; however, the difference was not significant ($P = 0.054$).

Preliminary determinations with mercury bichloride and ammoniated mercury were conducted with stainless steel penicylinders. However, the results on subsequent determinations became erratic, and these unusual inhibition zones were found to be due to mercury which had been adsorbed on the cylinders. Usual cleaning processes did not decontaminate the cylinders, and porcelain cylinders were used thereafter.

## CONCLUSIONS

Standard curves are neither too difficult nor too time consuming for use in routine ointment diffusion tests. The fallacy in comparing diffusion from ointments on a basis of inhibition zones alone has been pointed out, and it is recommended that the standard curve method be adopted to test the diffusion of antibacterial drugs from ointment bases.

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# Paper Chromatographic Evaluation of Rauwolfia Species*

By B. P. KORZUN, A. F. ST. ANDRÉ,† and P. R. ULSHAFER

**A method for the assay of Rauwolfia root material by paper chromatography is presented. A number of Rauwolfia species are evaluated for reserpine content and for the distribution of other alkaloids.**

THE INTEREST AROUSED by the unique biological activity of reserpine, an alkaloid first isolated from the Indian *R. serpentina* (1), has lead to an extensive investigation of the alkaloid content of other Rauwolfia species. Previous investigators have used partition chromatography on paper (2–4), and we have found this procedure, with the extensions and modifications outlined in this paper, to be of invaluable aid in the examination of the alkaloids present in the many root samples available to us. By a visual comparison of the chromatograms of root extracts with those produced by known concentra-

* Received April 23, 1957, from the Research Department, CIBA Pharmaceutical Products Inc., Summit, N. J.

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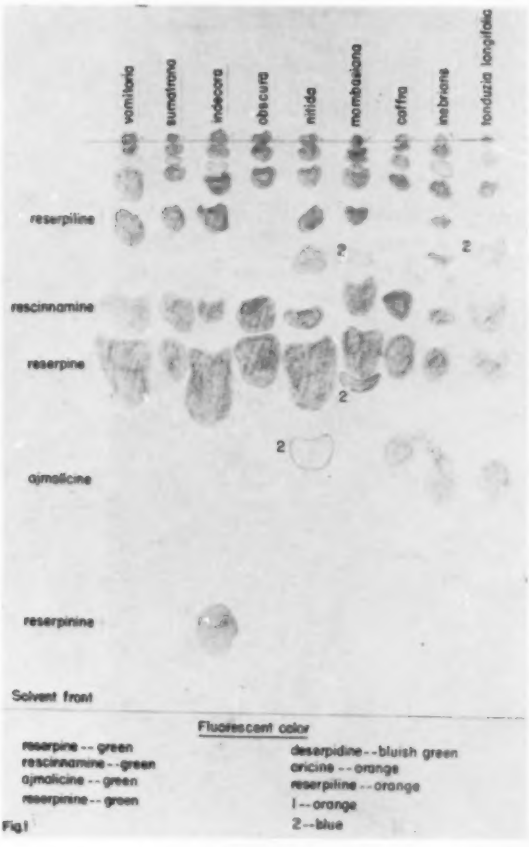


Figure 1

tions of pure alkaloids, we have been able to determine the alkaloidal distribution in some 25 Rauwolfia species (Figs. 1, 2, and 3).

## EXPERIMENTAL

**Preparation of Sample Solution and Extraction Procedure.**—The roots are ground with a Ball and Jewel No. 202 mill through a 1/32-inch screen. To 3 Gm. of ground root is added 2 ml. of water. After the root is thoroughly wetted, add 12 ml. benzene or enough to cover the roots and then reflux for one hour. The mixture is filtered hot, and washed with approximately 12 ml. of benzene. The above extraction is repeated a second time, and the combined extracts are evaporated to dryness *in vacuo*. The residue is transferred to a 5-ml. volumetric flask using chloroform as the solvent. This solution (*a*) is then spotted on the chromatograms for developing.

**Paper Chromatography.**—Reagents used were benzene (thiophene free), cyclohexane, and formamide 99% (Matheson, Coleman and Bell).

*Immobile phase*—Dilute 70 ml. of formamide with 30 ml. of methanol.

*Mobile phase.*—One part by volume of benzene and one part by volume of cyclohexane saturated with formamide.

**Chromatographic Procedure.**—Standard Chromatographic jars, set up for descending chromatography, are lined with filter paper. The mobile phase is placed in the solvent troughs and at the bottom of the chamber with the liner dipping into the solvent. The chamber is then allowed to become equilibrated.

Points for the application of the sample are marked 3 cm. apart on a line 6.5 cm. from the top of Whatman's No. 1 paper cut to 46-cm. lengths. The paper is immersed in the immobile phase and the excess solution is removed by passing the paper, sandwiched between two sheets of filter paper, through a hand wringer.

Extract (*a*) is spotted on the chromatograms in volumes 5, 10, and 15 λ. A freshly prepared reserpine, or other alkaloid standard, dissolved in chloroform is spotted on the chromatograms in varying concentrations from 1 to 14 γ in 1-γ increments. The diameter of the spots must be approximately the same. The chromatograms are then placed into the chambers and allowed to develop until the solvent front is about 3 cm. from the bottom (approximately five hours). The chromatograms are removed and dried at 90° for approximately ten minutes. The visual comparison is made with the aid of an ultraviolet lamp, under which light the spots fluoresce. The size and intensity of the fluorescence is taken into consideration in making the estimation. The fluorescence may be intensified for some alkaloids by spraying lightly with glacial acetic acid.

**Isolation Procedure for Reserpine.**—The procedure given is for a 50-Gm. sample. The method has been used successfully on samples as small as 15.0 Gm. using correspondingly smaller volumes of solvents.

To the ground root material, 300 ml. of methanol is added. The mixture is refluxed for one hour. The hot extract is filtered by decantation and the marc is washed twice by adding 100 ml. methanol, heating to boiling, and filtering by decantation. The combined filtrates are concentrated to 25 ml. and 25 ml. of fifteen per cent acetic acid is added. The solution is extracted twice with 25-ml. portions of ethylene dichloride. The ethylene dichloride extracts are combined and washed to pH 9 with 2*N* $Na_2CO_3$ (*ca.* 25 cc.) and then with water until neutral. Usually one or two 25-ml. water washes are sufficient. The extract is evaporated to dryness *in vacuo*. The solid residue is triturated with 5 ml. benzene, filtered, and washed with 5 ml. benzene. The filtrate is evaporated to dryness and *ca.* 0.5 ml. methanol is added. After cooling at *ca.* 5° overnight the reserpine crystals are filtered and washed with *ca.* 3 ml. methanol and weighed and identified.

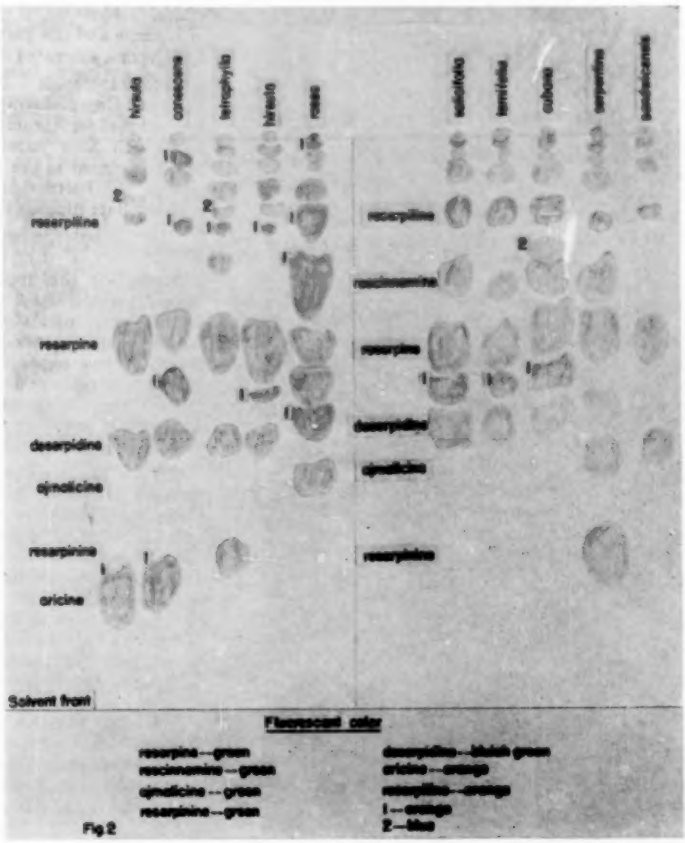


Figure 2

TABLE I.—RESERPINE CONTENT OF RAUWOLFIA SPECIES[a] BY PAPER CHROMATOGRAPHIC ASSAY

| Species | Reserpine, % | Species | Reserpine, % |
|---|---|---|---|
| *R. vomitoria* | 0.20 | *R. ligustrina* (*R. ternifolia*) | 0.03 |
| *R. sumatrana* | 0.04 | *R. salicifolia* | 0.07 |
| *R. obscura* | 0.05 | *R. cubana* | 0.04 |
| *R. ligustrina* (*R. indecora*)[b] | 0.15 | *R. serpentina* | 0.09 |
| *R. nitida* | 0.20 | *R. sandwicensis* | 0.02 |
| *R. mombasiana* | 0.05 | *R. paraensis* | 0.01 |
| *R. caffra* | 0.02 | *R. bahiensis* | 0.01 |
| *R. inebrians* | 0.01 | *R. sprucei* | 0.01 |
| *R. tetraphylla* (*R. hirsuta* – Central America) | 0.15 | *R. pentaphylla* (root bark) | 0.45 |
| | | *R. viridis* (*R. lamarkii*) | 0.03 |
| *R. tetraphylla* (*R. canescens* – India) | 0.05 | *R. littoralis* (*R. macrocarpa*) | 0.03 |
| *R. tetraphylla* | 0.08 | *R. grandiflora* (*R. affinis*) | ... |
| *R. rosea* | 0.04 | *Tonduzia longifolia* | 0.01 |

[a] The species names given in the table conform as far as is possible to those given in "A Revision of Rauvolfia with Particular Reference to the American Species," A. S. Rao, Annals of the Missouri Botanical Garden, **43**, 253(1956).
We are indebted to Prof. R. E. Woodson, Jr., Washington University, St. Louis, Mo., and to Dr. H. Hürlimann, CIBA Ltd., Basle, Switzerland, for the botanical identification of the plants examined.
[b] The second name given is the original identification of the specie.

## DISCUSSION

The above paper chromatographic method was applied to 25 Rauwolfia species in order to evaluate the reserpine content and the distribution of other alkaloids. An average deviation of ±10% from the isolated reserpine content was obtained. This range was considered acceptable due to the low concentration of reserpine, usually about 0.01% to 0.20%.

An attempt to elute the alkaloids from the paper

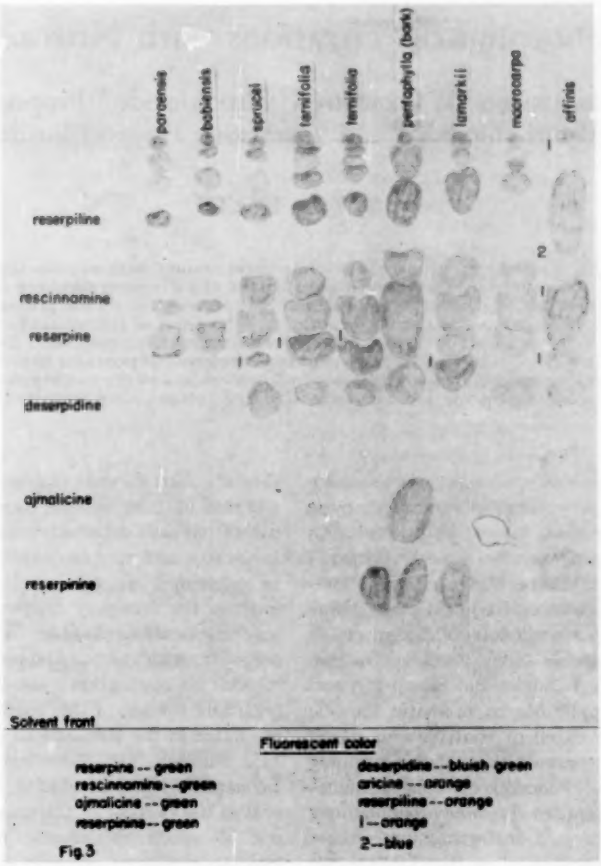


Figure 3

and to estimate their concentration by their ultraviolet spectra was unsuccessful.

Data obtained by application of the paper chromatographic assay procedures are listed in Table I. It is important to keep in mind that the concentration of any particular alkaloid varied considerably in different samples of the same species obtained from different geographical areas. Figs. 1, 2, and 3 show the chromatographic patterns of the Rauwolfia species. A number of species do not contain deserpidine but contain rescinnamine and the reverse holds for other species. Many species contain both rescinnamine and deserpidine.

## SUMMARY

A paper chromatographic method is presented for the assay and evaluation of crude root extracts with 3 grams of ground material from Rauwolfia species. An extraction procedure for the isolation of reserpine has been described.

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# Spectrophotometric Titrations with Nitrous Acid I.*

## The Estimation Of Procaine Hydrochloride,[1] Propoxycaine Hydrochloride;[2] and Tetracaine Hydrochloride[3]

By E. L. PRATT

**Spectrophotometric titrations of three aromatic amines with nitrous acid are described. Measurements made during the course of a given titration are dependent on the presence or absence of the nitrous acid spectrum, or on the presence or absence of diazo or nitroso derivative spectra. The point of intersection of straight lines of different slope, occurring prior to and after reaction completion, is a measure of amine equivalency. In particular, measurements of procaine hydrochloride, propoxycaine hydrochloride, tetracaine hydrochloride, and the combination of procaine hydrochloride with tetracaine hydrochloride are discussed.**

THE AMINE GROUP may be measured chemically in several ways. Because of its strong basic function in acetic acid, it may be titrated with good precision using perchloric acid. Mitchell (1) and Welsh (2) have presented techniques dependent on the acetylation of the amine group. The micro procedure of McIntire (3) applicable to aliphatic amines; and of Bratton and Marshall (4), Bandelin and Kemp (5), and St. John (6), applicable to aromatic amines; are among many excellent contributions which have made trace concentrations of this grouping readily accessible. Reactions of aliphatic amines with nitrous acid are not commonly found among routine procedures used in the measure of these substances, with exception, in the classical van Slyke method (7) involving a primary group. For many years diazotization and nitrosation procedures have been used in the estimation of aromatic amines. Macro methods of this type are frequently noted in official compendia (8, 9). These latter types of procedures are carried out by titrating a cold, acid solution with sodium nitrite until nitrous acid appears in detectable excess. Detection is generally facilitated with starch-iodide paste or paper.

It is common practice in titrimetry to use secondary reactions of chemical indicators, internal or external to the system being measured, as the means of detecting the end of a primary reaction. When one measures in this way, the single conclusion of primary reaction terminus is noted, with little, if any, insight being afforded the analyst during the course of the titration. This factor of insight has been gained, in varying degrees by adjoining a physical detector, which presents some measure of system change for each addition of titer volume, to the system. The use of physical detectors is not a new concept. It has appeared most frequently in the last decade as an applied concept, paralleling the development of the necessary instruments, which have made applications possible. The combination of titrimetry with spectrophotometry is one instance of such an application. General considerations regarding the use of this particular combination are found in the publications of Malmstadt (10, 11), Furman (12), Rowley (13), Zak (14), Leonard (15), and Goddu (16, 17), among others.

It is the purpose of this paper to describe the use of spectrophotometric titrimetry in the estimation of some aromatic amines with nitrous acid. Absorbance measurements made during the course of a given titration are dependent on the presence or absence of the nitrous acid spectrum, or on the presence or absence of diazo or nitroso derivatives spectra. The point of intersection of straight lines of different slope, occurring prior to and after reaction completion, is a measure of amine equivalency. In particular, measurements of procaine hydrochloride, propoxycaine hydrochloride, tetracaine hydrochloride, and the combination of procaine hydrochloride with tetracaine hydrochloride are discussed.

The substances procaine hydrochloride and propoxycaine hydrochloride are primary aromatic amines. They undergo the expected diazo reaction. Tetracaine hydrochloride, because its ring bound amine group is secondary, reacts with nitrous acid forming a nitroso derivative. Since the ring bound amine, in all three compounds, is in proximity with the structure primarily contributing to the electronic spectra, any alteration of this group is readily noted in an ultraviolet

* Received April 27, 1957, from the Control Laboratories, Winthrop Laboratories, Rensselaer, N. Y.

[1] Tradmark, Novocain®.
[2] Trademark, Ravocaine®.
[3] Trademark, Pontocaine®.

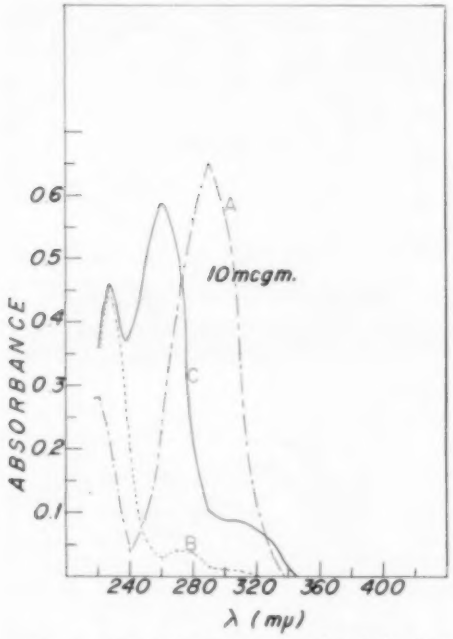


Fig. 1.—Ultraviolet spectra procaine derivatives, cell length 1.0 cm. A—procaine monohydrochloride; B—procaine dihydrochloride; C—procaine diazonium chloride.

spectrum change. The three compounds, procaine hydrochloride, tetracaine hydrochloride, and propoxycaine hydrochloride, in aqueous solution, all exhibit strong absorption bands in the region of 300 mμ. When aqueous solutions of these substances are acidified, the ring bound nitrogen becomes salted, and the bands in the region of 300 mμ diminish. Subsequent diazotization or nitrosation, occurring on addition of sodium nitrite to the dihydrochlorides in acid solution, presents a further influence on the absorbing structures. Spectra depicting these ultraviolet light absorbing systems are presented in Figs. 1, 2, and 3. It may be noted that the further influence, resulting when nitrosation of tetracaine dihydrochloride and diazotization of propoxycaine dihydrochloride begins, is indicated by appearance of new absorption bands enveloping a portion of the ultraviolet region including the region from 300 mμ to 400 mμ. The spectrum of procaine diazonium chloride does not extend beyond 346 mμ. Spectrophotometric titrations with sodium nitrite, carried out at 385 mμ, on acid solutions of tetracaine hydrochloride and propoxycaine hydrochloride, present absorbance data dependent on the formation of the nitroso

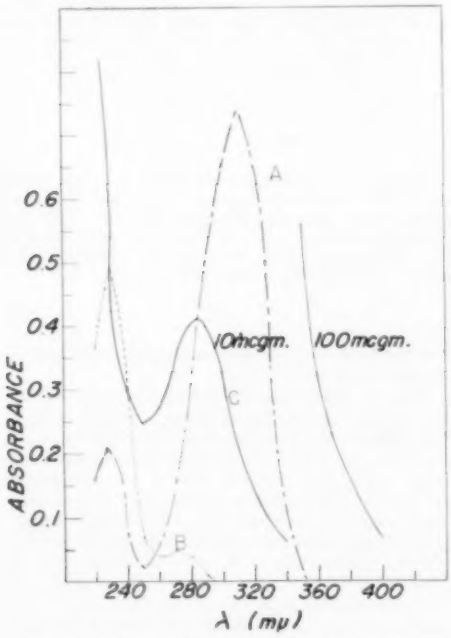


Fig. 2.—Ultraviolet spectra tetracaine derivatives, cell length 1.0 cm. A—tetracaine monohydrochloride; B—tetracaine dihydrochloride; C—tetracaine nitroso derivative.

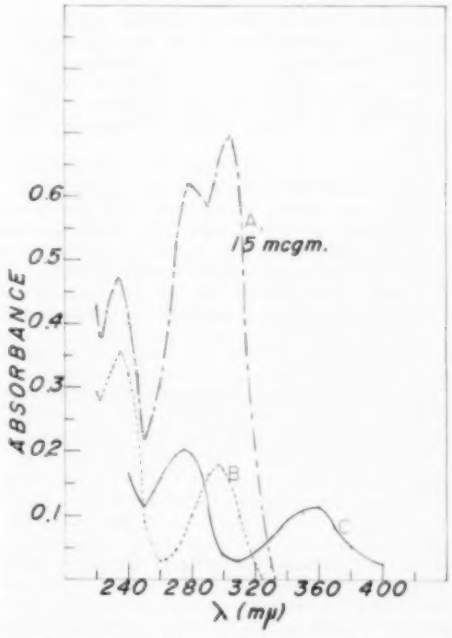


Fig. 3.—Ultraviolet spectra propoxycaine derivatives, cell length 1.0 cm. A—propoxycaine monohydrochloride; B—propoxycaine dihydrochloride; C—propoxycaine diazonium chloride.

and diazo derivatives, respectively. These derivatives may be detected as they are formed. A similar titration of procaine presents no significant absorbance at 385 mμ until it has been completely diazotized. At this point, however, free nitrous acid appears in the titrating system and this substance's 385-mμ absorption maximum becomes a detectable quality. The spectrum of nitrous acid is illustrated in Fig. 4.

While it is customary, primarily from the point of view of sensitivity, to measure spectrum intensity at band maxima, the factor of sensitivity is of only secondary importance here. Of primary interest is the measure of absorbance change per unit addition of titrant, where this can be done with reasonably narrow slit widths, using wide titration vessels of borosilicate glass.

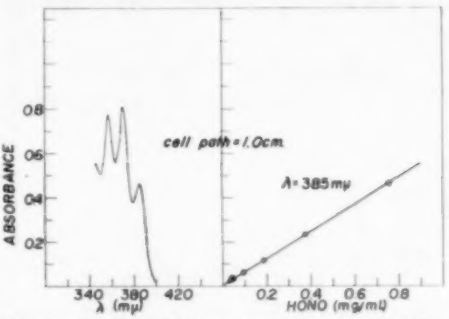


Fig. 4.—A—ultraviolet spectrum nitrous acid; B—absorbance *vs.* HONO concentration; λ = 385 mμ.

## EXPERIMENTAL

All titrations reported herein have been carried out at room temperature and in approximately 6*N* HCl. Ultraviolet spectra of the diazonium salt of procaine and the nitroso derivative of tetracaine do not suggest degradation has occurred over a sixty-minute period. Propoxycaine diazonium chloride is not as stable as procaine diazonium chloride, when stability is determined by timewise comparison of the spectra of these substances in strong acid solution at room temperature. This difference in stability, however, is not significant in so far as titrimetric measurements are concerned.

**Reagents and Apparatus**—Sodium nitrite (0.1 Normal) was standardized by titration against procaine hydrochloride. Volumetric dilutions of this solution were used without further standardization.

Additional reagents used were as follows: hydrochloric acid—6 Normal, sodium carbonate solution —10%.

Included in the apparatus were: "electrolytic" beakers, borosilicate glass—250 cc.; buret, standard—25 cc.; buret, ultramicro, "Gilmont"—1.0 cc.; magnetic stirring element (the magnetic element and shaft, after removal of cover of Magnetic Stirrer, Cat. No. J-711, Scientific Glass Apparatus Co., were used); stirring bar (an iron nail, 1-cm. long, sealed in a 1.5-cm. length of 5-mm. diam. borosilicate glass tubing).

The spectrophotometer used was a Beckman Model "B"—modified similar to that outlined by Goddu (17). The cell compartment base plate was replaced with one of aluminum and a hole was placed therein to permit passage of the shaft normal to a magnetic element. The spectrophotometer was positioned on two rectangular blocks elevating the instrument base plate $2^3/_4$ inches above table level. The magnetic element was extended just above the aluminum base plate but below the lowest guide rod of the cell carriage supplied with the instrument. This permitted interchange of the instrument cell holder and the rectangular aluminum titration compartment, noted in Fig. 5, without removal of the magnetic element. The titration compartment was set in place over the magnetic element and adhesive backed sponge rubber was used to prevent the passage of stray light between the closely fitted sides of the titration compartment and the walls of the instrument cell compartment.

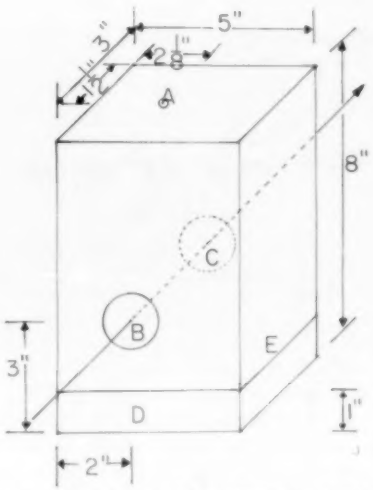


Fig. 5.—Titration chamber adapted to Beckman model B spectrophotometer. A—inset chamber cover, $^3/_8$-in. diameter for buret tip; B—light entrance aperture, $1^1/_2$ in.; C—light exit aperture, $1^1/_2$ in.; D—compartment for rotating magnetic element; E—titration vessel shelf.

**General Procedure.**—Titrations were carried out by initially placing the titration vessel, containing the substance to be measured in a total volume made to 150 cc. with 6*N* HCl, into the titration compartment. The glass encased stirring bar was placed in the titration beaker and the motor driven magnetic element was rotated to effect rapid stirring. The instrument in the number 2 sensitivity position, with wavelength set at 385 mμ, was adjusted to 0.0 absorbance after the titration vessel was positioned with the stirring cone out of the light path. The buret tip was placed through the opening provided in the compartment cover and permitted to extend just below the surface of the solution to be titrated. Titration was then begun and appropriate increment volumes of titrant were added. In the sub-

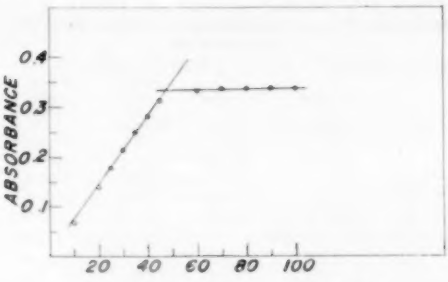


Fig. 6—Spectrophotometric titration propoxycaine monohydrochloride (1.5 mg.), cell 250-cc. electrolytic beaker, λ = 385 mμ.

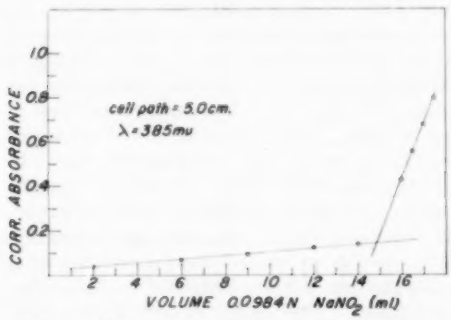


Fig. 7.—Spectrophotometric titration procaine monohydrochloride (399.0 mg.), cell 250-cc. electrolytic beaker, λ = 385 mμ.

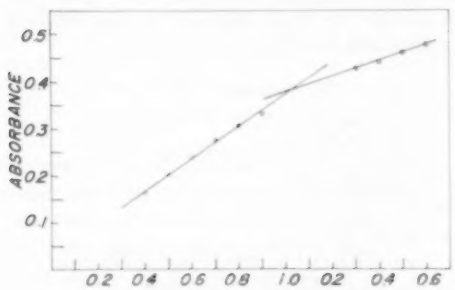


Fig. 8.—Spectrophotometric titration tetracaine monohydrochloride (30.0 mg.), cell 250-cc. electrolytic beaker, λ = 385 mμ.

sequent plotting of data, the intersection of straight lines obtained from data distant from the apparent point of intersection was used as the end point.

## APPLICATION

**Estimation of Propoxycaine Hydrochloride**—A sample equivalent to 1.5 mg. propoxycaine hydrochloride was transferred to the titration vessel and diluted with 6*N* HCl. Sodium nitrite (0.1 *N*) was added from a micro buret. The data of an actual titration are illustrated in Fig. 6 and results of repeated titrations of a single commercial sample are found in Table I.

TABLE I.—SPECTROPHOTOMETRIC TITRATION OF COMMERCIAL PROPOXYCAINE HYDROCHLORIDE

| Determination | Propoxycaine HCl Found mg./0.3 ml. | % of Label Claim | Precision[a] |
|---|---|---|---|
| 1 | 1.495 | 99.67 | |
| 2 | 1.497 | 99.80 | |
| 3 | 1.495 | 99.67 | |
| 4 | 1.504 | 100.27 | 0.29% |

[a] Estimated standard deviation.

**Estimation of Procaine Hydrochloride**—Crystalline procaine hydrochloride was transferred to a titration vessel and diluted with 6*N* HCl. Sodium nitrite (0.1 *N*) was added from a 25-cc. macro buret. The data of an actual titration are illustrated in Fig. 7 and results of three determinations are found in Table II. In a macro titration of this type it is necessary that corrected absorbance[1] values be plotted as ordinate in the graphic presentation of absorbance *vs.* volume data.

TABLE II.—SPECTROPHOTOMETRIC TITRATION OF PROCAINE HYDROCHLORIDE

| Procaine HCl Taken (mg.) | Procaine HCl Found (mg.) | Per cent of Theory Found | Precision |
|---|---|---|---|
| 446.2 | 446.3 | 100.02 | |
| 420.3 | 418.6 | 99.60 | |
| 410.4 | 412.0 | 100.39 | 0.4% |

**Estimation of Tetracaine Hydrochloride**—A sample equivalent to 30.0 mg. tetracaine hydrochloride was transferred to the titration vessel and diluted with 6*N* HCl. Sodium nitrite (0.1 *N*) was added from a micro buret. The data of an actual titration are illustrated in Fig. 8 and results of replicated measurements of one tetracaine hydrochloride solution are presented in Table III.

TABLE III.—SPECTROPHOTOMETRIC TITRATION OF TETRACAINE HYDROCHLORIDE (30.0-mg. SAMPLE THEORY)

| Detn. | Tetracaine HCl Found (mg.) | Per cent of Theory Found | Precision |
|---|---|---|---|
| 1 | 30.3 | 101.0 | |
| 2 | 30.2 | 100.3 | |
| 3 | 30.3 | 101.0 | |
| 4 | 29.7 | 99.0 | |
| 5 | 30.2 | 100.3 | 0.8% |

[1] Corrected Absorbance = Absorbance × $[(V_o + V)/(V_o)]$
$V_o$ = Volume prior to sodium nitrite addition.
$V$ = Volume of sodium nitrite added.

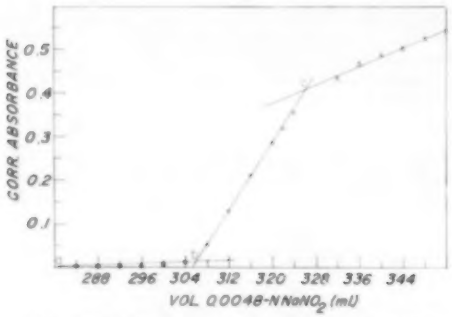


Fig. 9.—Spectrophotometric titration procaine monohydrochloride (400.0 mg.) and tetracaine monohydrochloride (30.0 mg.), cell 250-cc. electrolytic beaker, $\lambda$ = 385 m$\mu$.

**Estimation of Procaine Hydrochloride and Tetracaine Hydrochloride when Combined in Aqueous Formula**—A solution was prepared containing in each cubic centimeter: 20.0 mg. procaine hydrochloride, 1.5 mg. tetracaine hydrochloride, 0.1 mg. 3,4-dihydroxynorephedrine, and 2.0 mg. of a bisulfite derivative. (This formulation is comparable to one which has long been commercially available.[5]) Prior to the estimation of the anesthetic content, it was necessary to separate the procaine and tetracaine components from the sympathomimetic amine and bisulfite derivative. This was accomplished by transferring a portion of the prepared solution, equivalent to 400.0 mg. procaine hydrochloride, to a 125-cc. separatory funnel. Three cubic centimeters of sodium carbonate (10%) was added to the funnel and the solution therein was extracted with four 30-cc. portions of $CHCl_3$. The chloroform extracts were filtered through chloroform saturated cotton and the filtrates were combined in a 250-cc. separatory funnel. The chloroform was then extracted with five 30.0-cc. portions of 6$N$ HCl and the total acid extracts was collected in a 250-cc. "electrolytic" beaker. The stirring bar was placed into the beaker and exactly 14.0 cc. sodium nitrite (0.1 $N$) was slowly added while the solution was rapidly stirred. At this point the instrument was set at 0.0 absorbance and the general procedure, previously described, was followed. The titration with the beaker in the light path was continued using sodium nitrite (0.005$N$). Corrected absorbance measurements[6] were noted after each 4.0-cc. volume addition of titrant from: 0 to 20 cc., 28 to 40 cc., and 52 to 72 cc. The data of an actual titration are illustrated in Fig. 9 and results of repeated analyses of one solution are reported in Table IV. The procaine hydrochloride content is equivalent to the total of sodium nitrite consumed to point $B$, while the tetracaine hydrochloride content is equivalent to the sodium nitrite consumed from point $B$ to point $C$.

[5] Available as N. P. C. Solution from The Cook-Waite Laboratories.
[6] See footnote 4.

TABLE IV.—SPECTROPHOTOMETRIC TITRATION OF PROCAINE HYDROCHLORIDE AND TETRACAINE HYDROCHLORIDE

| Detn. | Procaine HCl Found mg. | Procaine HCl % of Theory | Tetracaine HCl Found mg. | Tetracaine HCl % of Theory |
|---|---|---|---|---|
| 1 | 403.9 | 100.9 | 29.3 | 97.7 |
| 2 | 400.3 | 100.1 | 29.7 | 99.2 |
| 3 | 400.1 | 100.0 | 30.6 | 102.0 |
| 4 | 400.6 | 100.2 | 29.9 | 99.6 |
| | Precision—0.407% | | Precision—1.78% | |

## DISCUSSION

The general approach presented for the measure of three particular aromatic amines permits measurement of these substances without the use of ice and starch-iodide type indicators. Instrumentation, suitable for measuring purposes, is relatively inexpensive and probably is available in most laboratories. The precision noted in the analyses carried out is less than one per cent in all instances, with exception, in the tetracaine measure of the procaine-tetracaine mixture. In the titration of the mixture of a primary aromatic amine (procaine hydrochloride) and the secondary aromatic amine (tetracaine hydrochloride), it has been demonstrated that diazotization takes place preferentially and completely before nitrosation begins. This finding, detectable in spectrophotometric titration, would not be realized if a reaction terminus indicator were used. Analytical time consumed for titrations of the type covered herein has been found to be equal to or less than the time required for conventional nitrous acid titrations.

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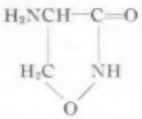
# Spectrophotometric Determination of Cycloserine and Isoniazid in Pharmaceutical Preparations*

By J. M. WOODSIDE, I. PIPER, and J. B. LEARY

**Cycloserine may be determined in certain dosage forms by dilution in hydrochloric acid and measurement of the absorbance at 219 millimicrons. Combinations of cycloserine with isoniazid may be determined from a single dilution from absorbances at 219 and 272 millimicrons, respectively. In this and in other dosage forms, values approaching the theoretical were obtained. The method requires no special apparatus and very little time. It shows good correlation with the microbiological method and is capable of following degradation in experimental preparations. The accuracy of the method is within ±2 per cent; its precision within ±1 per cent.**

CYCLOSERINE, AN ANTIBIOTIC produced by a strain of *Streptomyces orchidaceus* (1), has been reported to be useful in the treatment of severe pulmonary tuberculosis (2, 3) and certain type infections of the urinary tract (4). Its structure has been determined to be (1, 5):



D-4-amino-3-isoxazolidone

Jones (6) has described a method of analysis deemed suitable for cycloserine, alone, and in the presence of some interfering compounds. This method is based on the reaction of cycloserine with sodium nitritopentacyanoferroate in a slightly acid medium to give an intense blue-colored complex which may be quantitatively measured at 625 mμ. It has proved useful in analyses of fermentation broths, blood plasma, urine, and some pharmaceutical preparations.

Clinical investigations have indicated increased effectiveness when cycloserine is combined with isoniazid (7, 8), and mixtures of these two ingredients in capsule form have been developed.

In attempting to use the method of Jones (6) for the analysis of the cycloserine content of such a mixture, the isoniazid present was found to interfere, this interference inhibiting the development of the color complex in proportion to the amount of isoniazid present (Table I).

In the course of developing a method for isoniazid, ultraviolet absorption curves were obtained on 0.1 *N* hydrochloric acid solutions of isoniazid, cycloserine, and a combination of these two ingredients on the Cary recording spectrophotometer, (Fig. 1). These curves show absorption peaks for cycloserine at 219 mμ (*B*) and for isoniazid at 265 mμ and at 216 mμ (*A*).

It was observed that at 272 mμ, just slightly off the 265 mμ maximum, isoniazid exhibits

TABLE I.—ISONIAZID INTERFERENCE WITH JONES METHOD

| Solution | Cycloserine, mcg./ml. | Isoniazid, mcg./ml. | Absorbance, 625 mμ |
|---|---|---|---|
| A | 100 | None | 0.583 |
| B | 100 | 30 | 0.528 |
| C | 100 | 60 | 0.447 |
| D | 100 | 120 | 0.272 |

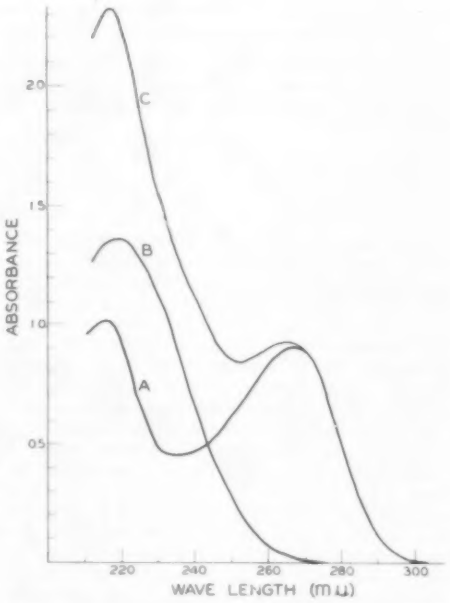



Fig. 1.—Absorbance curves—(Cary recording spectrophotometer); for solutions in 0.1*N* hydrochloric acid of: A—isoniazid 24 mcg. per ml., B—cycloserine 40 mcg. per ml., C—cycloserine 40 mcg. per ml., and isoniazid 24 mcg. per ml.

* Received March 29, 1957, from the Analytical Laboratories, Eli Lilly and Co., Indianapolis, Ind.
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Acknowledgment is due many members of the analytical staff for their helpful comments and to the Microbiological Testing Department for the microbiological assays performed.

considerable absorbance, while cycloserine shows none, and that at 219 mμ (the peak for cycloserine) the absorbance of the solution containing both of these compounds (*C*) appeared to equal the total of the individual absorbances (*A*) + (*B*).

## EXPERIMENTAL

To check conformance to Beer's Law, concentrations of cycloserine from 2 to 25 mcg. per ml. in 0.1 *N* hydrochloric acid and of isoniazid from 3 to 15 mcg. per ml. in 0.1 *N* hydrochloric acid were prepared. The absorbance measurements of each of these solutions was observed at 219, 265, and 272 mμ on a Beckman model DU spectrophotometer. All measurements at 219 mμ were made using a standard slit width setting of 1.8 mm. Data as shown in Tables II and III demonstrate conformance to Beer's Law for both compounds.

TABLE II.—CYCLOSERINE IN 0.1 *N* HYDROCHLORIC ACID

| | Concentration Cycloserine (mcg./ml.) 25 | 12.5 | 10 | 5 | 2 |
|---|---|---|---|---|---|
| $A_{219}$ mμ | 0.827 | 0.420 | 0.340 | 0.170 | 0.070 |
| $A_{265}$ mμ | 0.028 | 0.015 | 0.012 | 0.006 | 0.003 |
| $A_{272}$ mμ | 0.010 | 0.004 | 0.003 | 0.002 | 0.001 |

TABLE III.—ISONIAZID IN 0.1 *N* HYDROCHLORIC ACID

| | Concentration Isoniazid (mcg./ml.) 15 | 12 | 8 | 6 | 3 |
|---|---|---|---|---|---|
| $A_{219}$ mμ | 0.576 | 0.462 | 0.312 | 0.233 | 0.117 |
| $A_{265}$ mμ | 0.560 | 0.452 | 0.292 | 0.217 | 0.114 |
| $A_{272}$ mμ | 0.512 | 0.412 | 0.275 | 0.206 | 0.103 |
| Ratio $A219$ mμ to $A_{272}$ mμ | 1.13 | 1.12 | 1.13 | 1.13 | 1.13 |

From the preceding data, it is apparent that cycloserine exhibits negligible absorbance at 272 mμ and that for isoniazid, the ratio of the absorbance at 219 mμ to that at 272 mμ becomes a constant, [($A_{219}$ mμ/$A_{272}$ mμ) = 1.13]. Since cycloserine will dimerize under certain conditions (9), each production lot is controlled for dimer content. However, it has been ascertained that the amount of dimer thus tolerated is not sufficient to interfere with the proposed spectrophotometric method.

**Stability of Solutions of Cycloserine in 0.1 *N* Hydrochloric Acid.**—Cycloserine is stable in solutions of 0.1 *N* NaOH (6). Hence, for routine use, a solution of cycloserine standard containing 100 mcg. per ml. in 0.1 *N* NaOH may be prepared. For use in the described procedure, 10 ml. of this solution may be diluted to 100 ml. with 0.1 *N* hydrochloric acid in order to determine the standard absorbance of cycloserine at a concentration of 10 mcg. per ml. While this solution exhibits slow loss in absorbance (Table IV) upon standing, if the determination is made within thirty minutes after dilution, this loss is slight and may be considered negligible.

TABLE IV.—TIME VS. ABSORBANCE AT 219 mμ (CYCLOSERINE 10 MCG./ML. IN 0.1 *N* HYDROCHLORIC ACID)

| | |
|---|---|
| Initial | 0.341 |
| After 15 minutes | 0.341 |
| After 30 minutes | 0.340 |
| After 45 minutes | 0.337 |
| After 60 minutes | 0.336 |

A 10-mcg.-per-ml. solution of cycloserine standard in 0.1 *N* hydrochloric acid and a 6-mcg.-per-ml. solution of isoniazid in 0.1 *N* hydrochloric acid were prepared and the absorbance of each was determined at 219 mμ and at 272 mμ. In addition, the absorbance of a solution containing both 10 mcg. per ml. of cycloserine and 6 mcg. per ml. of isoniazid in 0.1 *N* hydrochloric acid was also determined. Absorbances observed are shown in Table V. In addition, various other ratios of isoniazid

TABLE V.—SOLUTION

| | *A*219 mμ | *A*272 mμ |
|---|---|---|
| Cycloserine 10 mcg./ml. | 0.341 | 0.002 |
| Isoniazid 6 mcg./ml. | 0.234 | 0.207 |
| Cycloserine 10 mcg./ml. Isoniazid 6 mcg./ml. | 0.575 | 0.210 |

and cycloserine were prepared and the solutions were observed in the above manner with results as shown in Table VI. Thus, it is readily observed that recovery of both components from known mixtures is satisfactory.

TABLE VI.—RECOVERY OF CYCLOSERINE AND OF ISONIAZID FROM SOLUTIONS CONTAINING VARIED AMOUNTS OF BOTH INGREDIENTS

| Solution | Cycloserine, Theoretical, mcg./ml. | Cycloserine, Found, mcg./ml. | Isoniazid, Theoretical, mcg./ml. | Isoniazid, Found, mcg./ml. |
|---|---|---|---|---|
| E | 10.0 | 9.9 | 6.0 | 6.0 |
| F | 8.0 | 7.9 | 4.0 | 4.0 |
| G | 6.6 | 6.6 | 8.0 | 8.0 |
| H | 13.3 | 13.3 | 4.0 | 4.0 |

## PROCEDURE

Dilute a suitable amount of sample in 0.1 *N* hydrochloric acid to an estimated 10 mcg. of cycloserine per ml. Determine the absorbance of this solution at 219 mμ and at 272 mμ on a Beckman DU spectrophotometer using 1-cm. matched silica cells and 0.1 *N* hydrochloric acid as a reference solution. For the measurement at 219 mμ, a standard slit width setting (1.8 mm.) is used.

To calculate isoniazid: (observed absorbance at 272 mμ)/[Standard absorbance (6 mcg./ml.) for Isoniazid at 272 mμ] × 6 = mcg./ml.

To calculate cycloserine: Observed absorbance at 272 mμ. × 1.13 = Isoniazid absorbance at 219 mμ. Observed absorbance at 219 mμ − Isoniazid absorbance at 219 mμ = Net absorbance at 219 mμ due to cycloserine. [Net absorbance at 219 mμ due to cycloserine/Standard absorbance (10 mcg./ml.) for cycloserine at 219 mμ] × 10 = mcg./ml. This

procedure has been applied to cycloserine and to dosage combinations. Results are shown in Tables VII and VIII.

TABLE VII.—COMPARISON OF METHODS AS APPLIED TO CYCLOSERINE AND CAPSULES CYCLOSERINE

| Item, Lot No. | Microbiological Assay[a] | Jones Method Assay | Spectrophotometric Method Assay |
|---|---|---|---|
| Cycloserine (Theoretical 1,000 mcg./mg.) (Results in mcg./mg.) | | | |
| 36 | 1,031 | 994 | 988 |
| 37 | 1,050 | 994 | 988 |
| 38 | 1,007 | 996 | 991 |
| 39 | 1,069 | 1006 | 982 |
| Capsules Cycloserine, 250 mg. (Results in mg./capsule) | | | |
| 83 | 255 | 251 | 251 |
| 84 | 245 | 238 | 239 |
| 10 | 252 | 262 | 254 |
| 11 | 256 | 263 | 262 |

[a] Turbidimetric assay using *Klebsiella pneumoniae* P.C.I. 602.

TABLE VIII.—COMPARISON OF MICROBIOLOGICAL AND SPECTROPHOTOMETRIC METHODS

| Lot No. | Microbiological Assay Cycloserine | Spectrophotometric Method Cycloserine | Isoniazid |
|---|---|---|---|
| Capsules Cycloserine 250 mg. and Isoniazid 150 mg. (Results in mg./capsule) | | | |
| 6 | 252 | 251 | 151 |
| 7 | 254 | 256 | 150 |
| 26 | 255 | 256 | 151 |

The procedure has also been applied to experimentally deteriorated combinations and appears to correlate with loss of cycloserine microbiological activity, although the microbiological loss appears to be somewhat greater than that shown by the spectrophotometric method, Table IX.

TABLE IX.—COMPARISON OF METHODS IN EXPERIMENTALLY DETERIORATED SAMPLES CONTAINING CYCLOSERINE AND ISONIAZID

| Sample | Spectrophotometric Method (Cycloserine in mg./Gm.) | Microbiological Assay Theoretical (520 mg./Gm.) |
|---|---|---|
| J | 468 | 474 |
| K | 349 | 276 |
| L | 464 | 476 |
| M | 185 | 133 |
| V | 514 | 495 |
| W | 463 | 449 |
| X | 360 | 335 |
| Z | 173 | 35 |

**Other Mixtures Containing Cycloserine.**—Other experimental mixtures of cycloserine in bases immiscible with water were prepared. An attempt was made to assay these samples by dissolving the base in ether and extracting the cycloserine with 0.1 *N* NaOH preparatory to using the Jones method. The alkali, however, caused difficulty in extraction due to some saponification and emulsification and thus, upon dilution of the extracted solutions, erroneously high values were obtained due to interfering substances. Extraction of the same samples with 0.1 *N* hydrochloric acid, however, did not cause emulsification and subsequent dilutions of the acid extractions and determination of the absorbance values at 219 mμ, in the manner described previously, yielded results that approached the theoretical values for these mixtures.

Similar type preparations in bases which were water soluble were assayed by making dilutions to 100 mcg. per ml. in 0.1 *N* NaOH for the Jones method, and to 10 mcg. per ml. in 0.1 *N* hydrochloric acid for the spectrophotometric method. No difficulty was encountered with either of the procedures and the results obtained were in fairly close agreement.

## SUMMARY

A method has been described by which cycloserine may be determined in certain pharmaceutical preparations. This method is based on dilution of a sample in 0.1 *N* hydrochloric acid with subsequent measurement of the absorbance at 219 mμ. Combinations of cycloserine with isoniazid may be determined from a single dilution of the sample material by measurement of the absorbance at 219 mμ and at 272 mμ.

In other experimental dosage forms, values approaching the theoretical were obtained. In such instances the dilution to 10 mcg. per ml. was advantageous in that interfering substances were diluted to a minimum.

The method is advantageous in that it requires no special reagents and consumes little time. It has shown correlation with the microbiological assay and appears to follow degradation in experimental samples. The method has an accuracy of within ±2% with a precision within ±1%.

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# A Dilatometric Study of Melting Behaviors of Some Fats, Waxes, and Related Substances of Pharmaceutical Importance I.*

## Polyethylene Glycols, Spermaceti, Cetyl Alcohol, Petrolatum, White Wax, and Theobroma Oil

By LOUIS J. RAVIN† and T. HIGUCHI

**A dilatometric study of the phase behavior of several compounds and mixtures commonly used in pharmaceutical systems has shown that a considerable amount of structural information concerning these systems can be obtained by this technique. Experimental data indicate that polyethylene glycol 4000, polyethylene glycol 6000, polyethylene glycol 20,000 and a mixture of polyethylene glycol 4000 and polyethylene glycol 6000 possess similar crystal structures in their solid states, the molecular components apparently forming nearly ideal solutions in both the melt and in the solid state. Polyethylene glycol ointment base, according to these measurements, contains much higher solids content than petrolatum of nearly the same consistency. Additional information concerning the melting behavior of spermaceti, cetyl alcohol, a mixture of spermaceti and cetyl alcohol, a mixture of spermaceti and polyethylene glycol 4000, a mixture of cetyl alcohol and polyethylene glycol 4000, and white wax, and the effect of rapid cooling on the phase forms of white petrolatum, theobroma oil, and polyethylene glycol 1500 is presented.**

FATS, WAXES, AND RELATED SUBSTANCES have been, and are important ingredients of many pharmaceutical products. Many have been found useful as emulsifying agents, others have been utilized to give consistency to various ointments, and a few have been used as lubricating agents in tablet manufacturing. Some of these have also been employed as coating agents for certain delayed-action drugs or as vehicles for injectable dosage forms. Waxes and fats, furthermore, have been widely employed as constituents in suppository bases.

The present investigation is concerned with the application of dilatometry to the study of phase transitions occurring in fats, waxes, and related substances important to pharmacy. The feasibility of this technique for determining quantitatively the extent of melting as a function of temperature and for detecting and measuring solid-solid transitions involving metastable forms are presented in this work. Particular emphasis has been placed on the melting behavior of some compounds and mixtures commonly used as stiffening agents in many pharmaceutical systems and on the effect of rapid cooling on the phase forms of white petrolatum, theobroma oil, and polyethylene glycol (PEG) 1500.

No attempt is made here to review the theories and general techniques concerning dilatometry as a considerable body of literature has already been built up in the field of edible fats and oils (1–6).

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### BEHAVIOR OF INDIVIDUAL SYSTEMS

**Polyethylene Glycols.**—The melting behaviors of several PEG compounds are shown in Figs. 1 and 2. In these plots the apparent specific volumes of the waxes were graphed as functions of the bath temperature. At the lowest temperature readings the substances under test were largely solids and any increase in volume was due almost entirely to the cubical expansions of the solid structure, an increase which was apparently linear with temperature over short ranges. As the temperature was raised, the systems experienced gradual melting, the sharpness of the melting being, as usual, dictated by the apparent purity of the systems. The melting phenomenon is evidenced in these curves by a significant departure from the original linear expansion. The termination of the melting process is indicated by a sharp break into another straight line corresponding to the cubical expansion of the now completely liquid system.

The polyethylene glycols are condensation products of ethylene oxide and water having a varying number of ethylene oxide molecules in the chain. For example, PEG 4000 has an average number of 66 ethylene oxide molecules in the chain and an average molecular weight of 4000. The dilatometric curves obtained for these compounds indicate, however, that they are relatively pure despite the known variations in the chain lengths of the molecular components. A summary of the dilatometric behavior exhibited by the polyethylene glycols is given in Table I. The melting ranges of PEG 4000 and PEG 6000 were especially small, probably because various

molecular weight components formed nearly ideal mixtures in both the melt and in the solid state. In the case of PEG 20000 there was a much wider melting range indicating either that the chain length varied a great deal or the presence of functional impurities.

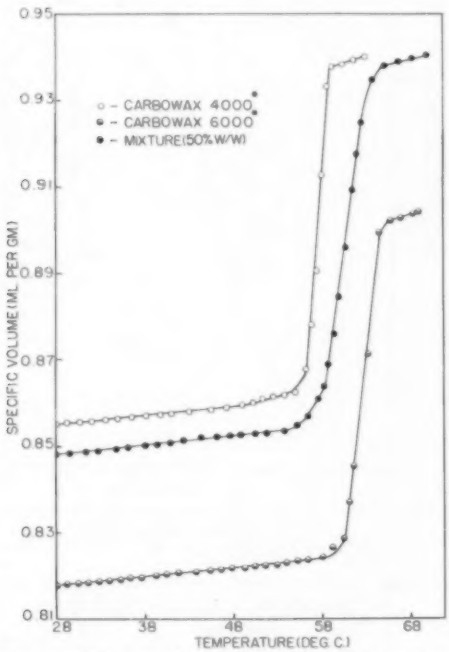



Fig. 1.—Dilatometric curves showing the melting dilation of several Carbowaxes.

* Trademark name, Carbide and Carbon Chemicals Co.

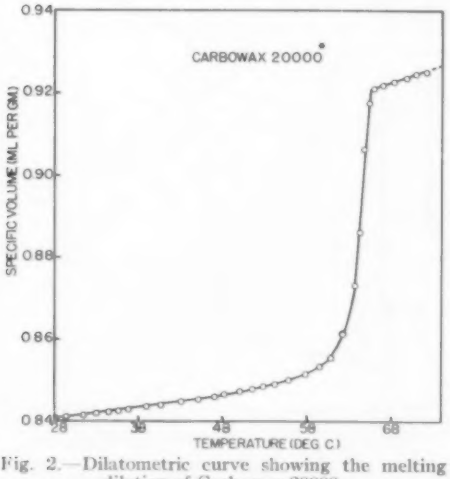



Fig. 2.—Dilatometric curve showing the melting dilation of Carbowax 20000.

* Polyethyleneglycol 20000, Dow Chemical Co.

TABLE I.—THE MELTING DILATIONS AND MELTING RANGES OF SEVERAL POLYETHYLENE GLYCOLS

| Compound | Melting Range, °C. | Melting Dilation ml./Gm. | Final M. p., °C. |
|---|---|---|---|
| PEG 4000 | 55–58.6 | 0.0752 | 58.6 |
| PEG 6000 | 59–64.8 | 0.0744 | 64.8 |
| PEG 20000 | 54–65.6 | 0.0684 | 65.6 |
| Equimixture of PEG 4000 & 6000 | 55–63.6 | 0.0776 | 63.6 |

It is interesting to note that an equimixture of PEG 4000 and PEG 6000 starts to melt at the same temperature that PEG 4000 begins to melt and completes melting approximately at the temperature that PEG 6000 melts. There is no significant depression of the melting point of PEG 6000. The similarity of the dilatometric curves probably occurs because the polyethylene glycols have a similar coefficient of expansion and are miscible in all proportions. The melting dilation appears to be fairly constant for PEG 4000, PEG 6000, and the mixture; however, it decreases for PEG 20000. This may indicate that the melting dilation for the polyethylene glycols decreases as the molecular weight increases. This is opposite to the behavior of the glycerides whose melting dilation increases with an increase in molecular weight.

**Cetyl Alcohol and Spermaceti.**—The dilatometric curves obtained for cetyl alcohol, spermaceti, and an equimixture of both are shown in Figs. 3, 4, and 5. A summary of their dilatometric behavior can be seen in Table II. Spermaceti, obtained from the head of the sperm whale, is composed largely of cetyl esters of several long-chained fatty acids. This may account for the behavior exhibited when spermaceti is melted. The curve does not have any detectable inflection points which are often found for mixtures. Cetyl alcohol, on the other hand, obtained by the saponification of spermaceti, yields a curve composed of what appears to be several well defined inflection points.

An equimixture of spermaceti and cetyl alcohol causes a lowering of the original and final melting points of both compounds. The dilatometric curve resembles the curve obtained for spermaceti in that there are no detectable transition points. The melting dilation of the mixture is approximately the same as the melting dilation of spermaceti. This may indicate that the components are miscible in all proportions.

**Mixture of Spermaceti and PEG 4000, and Cetyl Alcohol and PEG 4000.**—An equimixture of spermaceti and PEG 4000, and cetyl alcohol and PEG

TABLE II.—THE EXPANSIBILITY AND MELTING RANGE OF CETYL ALCOHOL, SPERMACETI, AND THEIR MIXTURE

| Compound | Melting Range, °C. | Melting Dilation ml./Gm. | Final M. p., °C. |
|---|---|---|---|
| Cetyl alcohol | 35–49.2 | 0.1318 | 49.2 |
| Spermaceti | 35–48.2 | 0.1164 | 48.2 |
| Equimixture of spermaceti and cetyl alcohol | 32–42.2 | 0.1160 | 42.4 |

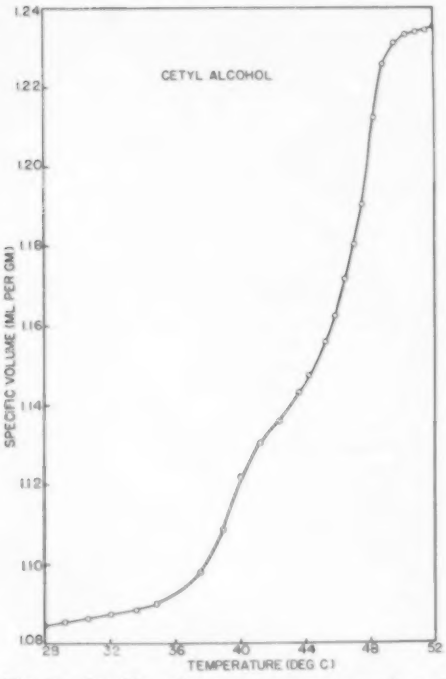



Fig. 3.—Specific volume *vs.* temperature of cetyl alcohol.

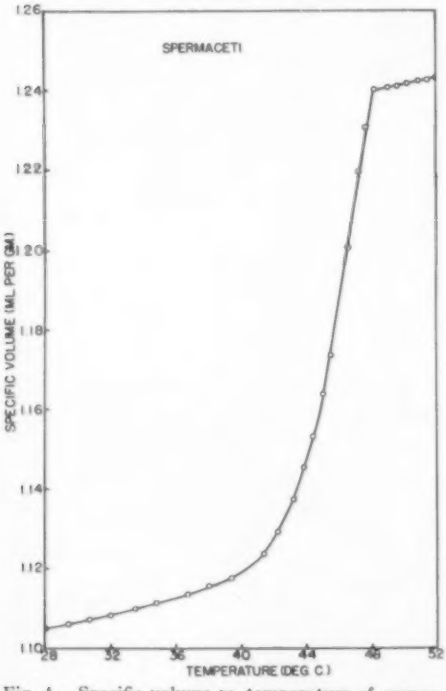



Fig. 4.—Specific volume *vs.* temperature of spermaceti.

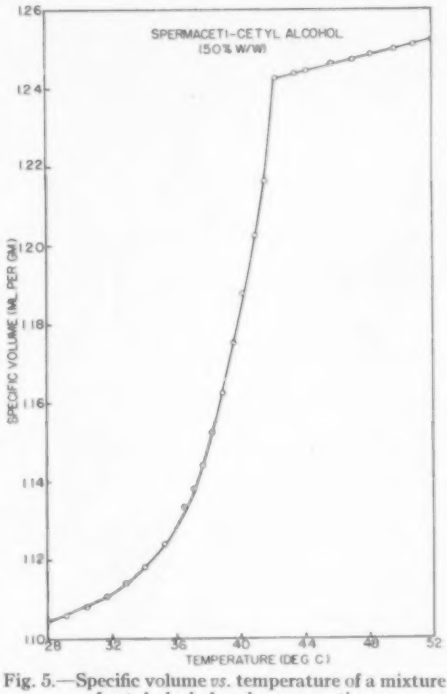



Fig. 5.—Specific volume *vs.* temperature of a mixture of cetyl alcohol and spermaceti.

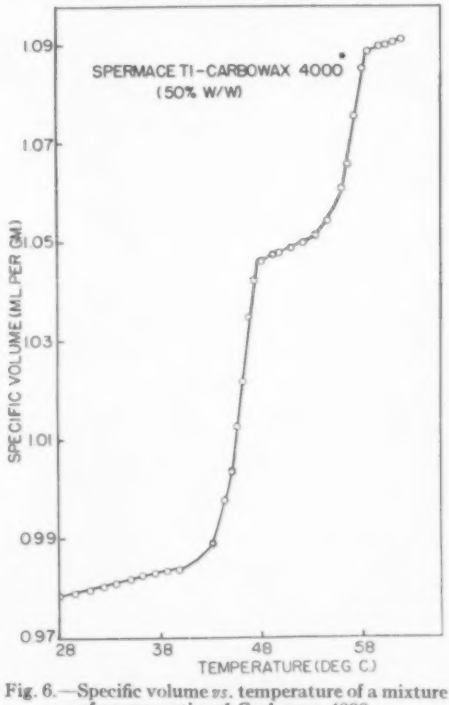



Fig. 6.—Specific volume *vs.* temperature of a mixture of spermaceti and Carbowax 4000.
*Trademark, Carbide and Carbon Chemicals Co.

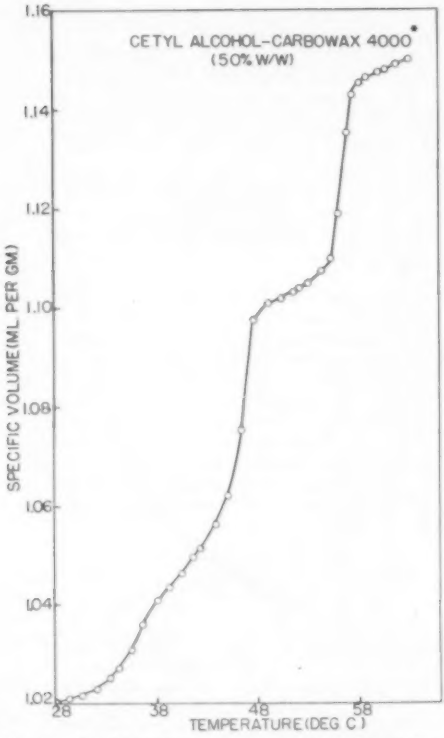



Fig. 7.—Specific volume *vs.* temperature of a mixture of cetyl alcohol and Carbowax 4000.

*Trademark name, Carbide and Carbon Chemicals Co.

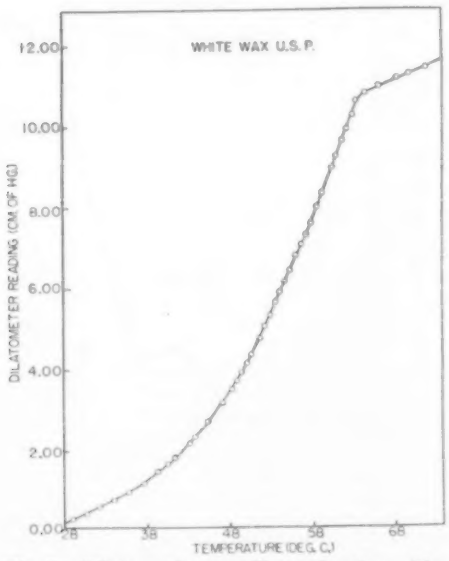



Fig. 8.—Dilatometric curve illustrating the melting behavior of white wax.

4000 exhibited a melting behavior unlike any previously obtained in this study. It is apparent from their dilatometric curves shown in Figs. 6 and 7 that the components melted independent of one another and their individual melting points were not affected to any great extent. Upon further analysis of the curves, it was found that the expansion of the individual components in a 50-50 mixture was approximately one half the expansion obtained when the compounds were melted alone. This apparently indicates that the components are completely immiscible and their melting dilations are not affected to any appreciable extent by the mixing.

**White Wax.**—Figure 8 is an illustration of the dilatometric behavior of white wax. The curve exhibits a wide melting range indicating the presence of some liquid phase even at room temperatures. The thermoplastic behavior is probably due to the softening and lubricating effect of the liquified component. The expansibility of the material is similar to that of the glycerides. This is not surprising since white wax is composed largely of long chained carboxylate esters.

## EFFECT OF RAPID COOLING ON THE PHASE COMPOSITION

**White Petrolatum.**—It is evident from Fig. 9 that white petrolatum exhibits only a small melting dilation. This is probably due to the presence of a high proportion of the liquid phase in white petrolatum even at low temperatures. There is proportionally very little solid in the system. Sudden cooling, which often produces unstable modifications, appears to have very little residual effect on petrolatum. It is clearly indicated from the dilatometric curve that no unstable forms were produced by the shock cooling procedure. There is a

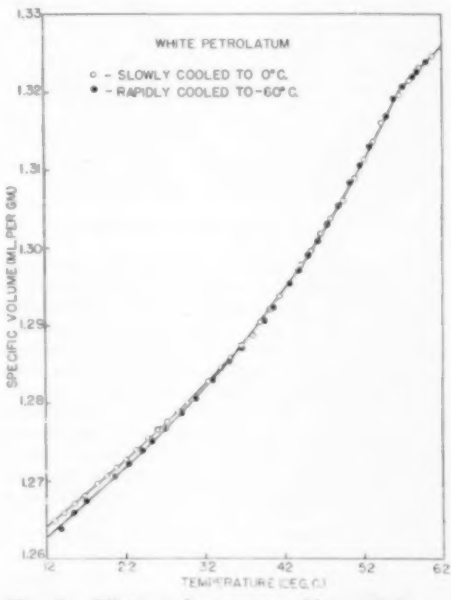



Fig. 9.—Dilatometric curves, white petrolatum, slowly and rapidly cooled.

slight lowering of the specific volume in the lower region of the curve. This may indicate that some of the liquid components were solidified by the sudden cooling which would account for the decrease in the specific volume. The dilatometric curve was plotted starting at 12°, since there was no significant difference in the slope of the line below that temperature.

**Polyethylene Glycol 1500.**—Figure 10 illustrates the effect of slow and rapid cooling on the phase behavior of PEG 1500. Although PEG 1500 is said to have the consistency of white petrolatum, it exhibits a much greater melting dilation. This is evident from a comparison of their dilatometric behavior. The greater melting dilation exhibited by PEG 1500 may be explained on the basis that it probably contains a higher percentage of solid components than the petrolatum sample. Rapid cooling of the sample did not produce any unstable forms; however, there was a more pronounced lowering of the specific volume in the lower temperature region of the curve. This is probably due again to the solidification of some of the liquid components present in PEG 1500.

**Theobroma Oil.**—The phase behavior of theobroma oil after being subjected to slow and rapid temperature changes is shown in Fig. 11. Unlike white petrolatum and PEG 1500, the sudden cooling has a pronounced effect on the dilatometric curve obtained. The sample which was slowly cooled showed no unusual behavior. The same sample rapidly cooled showed an expansion between 16° and 20°, and a contraction between 20° and 24°. The presence of an unstable modification probably caused this behavior. The sudden expansion followed by the contraction is due to the conversion of the less dense unstable form into the more dense stable modification. This behavior can be expected in all pharmaceutical systems that utilize theobroma oil that has been subjected to sudden changes in temperature. One important observation can be made from the dilatometric curve. The polymorphic transformation to the more stable form takes place between 20° and 24°, which is slightly below room temperature. If the finished products are allowed to stand at room temperature for any length of time, it is possible that complete conversion to the more stable form may take place.

## GENERAL DISCUSSION

It is evident from the data presented that dilatometry is an extremely useful tool in determining and understanding phase characteristics of high molecular weight waxy and fatty substances employed in pharmacy. Because of its great sensitivity and reproducibility it can serve, for example, as a control method for these difficult-to-characterize materials. Formation of solid solution among pharmaceutical components and of immiscible phases can be readily detected in pastes, bases, and other semi-solids. It should prove, furthermore, very useful in elucidating the solid state structures of many of these complex systems.

## EXPERIMENTAL

**Reagents.**—The following reagents were used: PEG 1500, PEG 4000, PEG 6000, PEG 20000, spermaceti, cetyl alcohol, white wax, U. S. P.; white petrolatum, U. S. P.; theobroma oil, U. S. P.; and mercury, triple distilled.

**Apparatus.**—Dilatometers, volumetric (2 mm. bore); vacuum pump; and optic micrometer.

The dilatometer used in this study is shown in Fig. 12. It is similar to the one described by Bailey (3) except for a few minor modifications. The

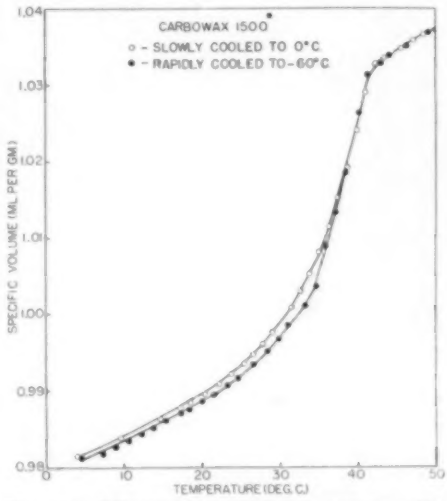



Fig. 10.—Dilatometric curves, Carbowax 1500, slowly and rapidly cooled.

*Trademark name, Carbide and Carbon Chemicals Co.

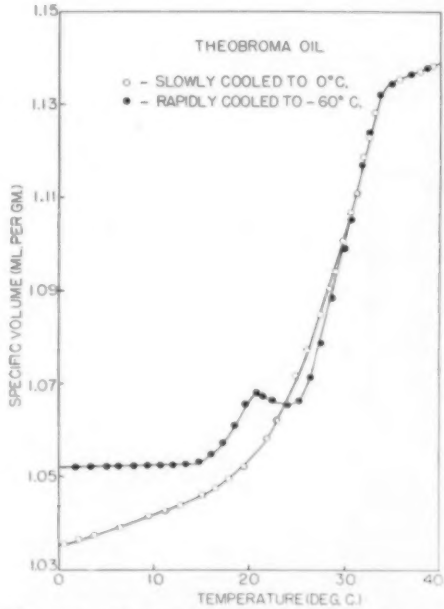


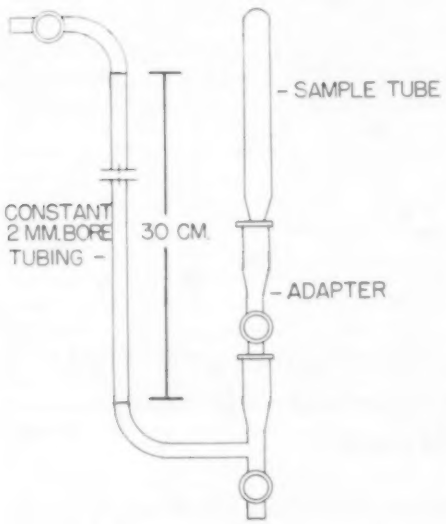

Fig. 11.—Dilatometric curves, theobroma oil, slowly and rapidly cooled.

sample tube was detachable to facilitate filling. An adapter was placed between the sample tube and the constant bore tubing to prevent sample from obstructing the constant bore tubing during the filling process.

**Calibration of the Constant Bore Tubing.**—The constant bore tubing of the dilatometer was filled with mercury. Known heights of mercury were taken from the dilatometer and weighed. The weight of the mercury was converted to volume and the radius of the tube was calculated. The capillary tubing used in all dilatometers had a constant bore of 2 mm. This value was used in all subsequent calculations.

**Preparation of the Mixtures.**—Approximately 1.5 Gm. of each ingredient was weighed into a suitable container. The contents were heated slowly in a water bath until complete melting took place. The container was taken out of the water bath and the contents allowed to cool slowly with constant stirring until complete solidification took place and a homogeneous mixture was obtained.

**Filling the Dilatometer.**—Since samples whose past history was important and samples whose past history was insignificant were studied, the following




VOLUMETRIC DILATOMETER
Figure 12

procedures for filling the dilatometers were adopted.

Procedure 1 (for samples whose past history was important)—approximately 1.5 Gm. of the compound to be studied was weighed into the sample tube. The dilatometer was assembled and attached to a vacuum pump. The sample was degassed overnight prior to filling the dilatometer. The outlet leading to a mercury reservoir was opened allowing the mercury to enter the dilatometer. When it was completely filled, the stopcock was closed and the pressure was allowed to rise slowly until normal atmospheric pressure was reached. The dilatometer was then ready for use.

Procedure 2 (for samples whose past history was insignificant)—approximately 1.5 Gm. of the sample was weighed into the sample tube. The dilatometer was assembled. The sample tube portion of the dilatometer was placed in a water bath which was sufficiently heated to insure complete melting of the compound. The capillary portion of the dilatometer was connected to a vacuum pump. The system was degassed, while the dilatometer was being rotated, to allow the sample to solidify on the outer walls of the sample tube. After complete solidification the dilatometer was filled with mercury as previously described.

**Procedure for Making a Dilatometric Study.**—The completely assembled dilatometer was placed in a water bath set at 28° and allowed to equilibrate overnight. The temperature was raised slowly at a rate of 6° per hour with readings being taken at intervals of 0.6°, using the optic micrometer. Readings were taken over a temperature range from 28° to a few degrees beyond the melting point of the sample being studied. If the sample had not been previously melted in the filling process, then the molten material was allowed to cool slowly to 28° and the melting process repeated. In this manner it was possible to detect the effect of remelting on the phase behavior of the sample.

The following variation in the procedure for making the dilatometric study was adopted for white petrolatum, PEG 1500, and theobroma oil. The completely filled dilatometer was placed in a water bath and cooled slowly to 0°. The temperature of the water bath was raised slowly with readings being taken at 0.6° intervals until complete melting had taken place. At this point the dilatometer and its contents were cooled suddenly in an acetone-dry ice bath and allowed to equilibrate for one-half hour. The dilatometer was transferred to a water bath held at 0° and allowed to equilibrate overnight. The temperature was raised slowly, as previously described, until melting took place.

**Calculation of the Specific Volume.**—The readings recorded during the dilatometric study represent the expansion of the sample being analyzed and the confining fluid. In order that the melting dilation of the sample be clearly defined, it is necessary to correct all readings for the expansion due to the confining fluid. This was done in the following manner.

The original readings recorded in cm. of mercury were converted to volume. The expansion due to the confining fluid, mercury in this case, was subtracted from each reading. The change in volume per temperature rise was added progressively to the original volume of the sample. The volume at the various temperatures was divided by the weight of the sample being studied. This resulted in the specific volume of the sample at a series of temperatures. The specific volume plotted as a function of temperature results in a typical dilatometric curve.

The original volume of the sample at 28° was calculated in the following manner: the weight of mercury in a completely filled dilatometer minus the weight of mercury in a dilatometer containing sample, divided by the density of mercury at 28°, results in the original volume of the sample.

All specific volume calculations were carried out

using the IBM 650 automatic calculator in the Numerical Analysis Laboratory at the University of Wisconsin.


## SUMMARY

Dilatometric curves between 28° and a few degrees beyond their melting points have been obtained for the following compounds and mixtures: PEG 4000, PEG 6000, PEG 20000, a mixture of PEG 4000 and PEG 6000, spermaceti, cetyl alcohol, a mixture of spermaceti and cetyl alcohol, a mixture of spermaceti and PEG 4000, a mixture of cetyl alcohol and PEG 4000, and white wax.

The behavior of the polyethylene glycols differed from the other mixtures studied in that there were no well defined points of inflection. A possible explanation for this may be that polyethylene glycols have similar coefficients of expansion and are miscible in all proportions. When two immiscible substances, spermaceti and PEG 4000, are mixed, they melt independently and their mixing has a very slight effect on the final melting point of the individual components. Contrary to this, when two miscible substances, spermaceti and cetyl alcohol, are mixed, they do not melt independently and their mixing has a pronounced effect on the final melting point. The individual melting points of the components are not detected in this case.

Sudden cooling did not appear to lead to the formation of metastable forms in the case of white petrolatum and polyethylene glycol. Rapid cooling, on the other hand, produced unstable forms very readily in theobroma oil. The dilatometric results obtained indicated that the transition to the more stable form for this material took place below room temperature.

The dilatometric information obtained from this study may be useful in determining why many ointment preparations undergo gradual changes in their physical appearance and rheological properties, why fat containing enteric coatings may change on standing, and why suppositories which have been cooled too rapidly exhibit anomalously low melting points.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(4) Singleton, W. S., and Gros, A. T., *J. Am. Oil Chemists' Soc.*, **29**, 149(1952).
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# Isonicotinoyl Hydrazine Derivatives and Their Activity against *Mycobacterium tuberculosis* in the Mouse*

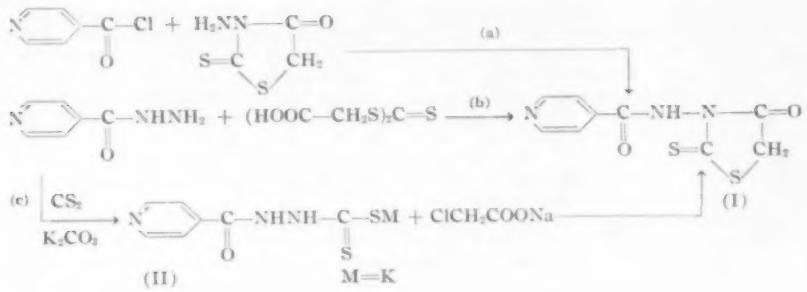
By RICHARD E. STRUBE and KURT F. STERN

**Four sulfur-containing isonicotinoyl hydrazine derivatives have been synthesized and evaluated against *Mycobacterium tuberculosis*, H37Rv, in mice. In this system, zinc and antimony 3-isonicotinoyl dithiocarbazinate, although highly active, are not superior to isonicotinoyl hydrazine.**

THE MOST active synthetic product at present available for combating tuberculosis is isonicotinoyl hydrazine and a few of its derivatives (1). Interest in the chemistry of 3-aminorhodanines (2) prompted the synthesis and evaluation of 3 - (4 - pyridinecarboxamino)rhodanine (I), which can be considered to be a combination of isonicotinoyl hydrazine and 3-aminorhodanine.

The present communication describes the preparation and antitubercular evaluation of 3-(4-pyridinecarboxamino)rhodanine (I) and a few salts of isonicotinoyl dithiocarbazinate (II, M = K, Zn, Sb). This compound can be prepared by methods (a), (b), and (c), which are outlined as follows.

* Received March 6, 1957, from the Research Division, The Upjohn Company, Kalamazoo, Mich.



Because of the ready availability of the starting materials and added simplicity, model studies were first carried out in the benzene series to determine the best of the three synthetic routes (a), (b), or (c). From this study, it was evident that the rhodanine derivative could be synthesized best by reacting trithiocarbodiglycolic acid with isonicotinoyl hydrazine [method (b)]. Although the preparation of the rhodanine nucleus by this method has been known for many years (3), its application to acid hydrazides has not yet been reported.

Potassium 3-isonicotinoyl dithiocarbazinate (II) was prepared from isonicotinoyl hydrazine, carbon disulfide, and anhydrous potassium carbonate according to the method of König, Siefken, and Offe (4), and Young and Wood (5). The corresponding antimony and zinc salts were obtained from the potassium salt by treatment with antimony or zinc chloride. The evaluation of these salts was of interest because these compounds contain both the isonicotinoyl moiety and the biologically active dithiocarbamate group. Each of these groups is present in certain systemically active antibacterials, such as isonicotinoyl hydrazine, hydroxymethyl isonicotinamide (6), and diethylaminodithiocarbamate (7, 8).

Although these sulfur derivatives of isonicotinoyl hydrazine are less toxic than the parent compound, none of them turned out to be superior when evaluated in mice by oral and subcutaneous routes. The most active compound, zinc 3-isonicotinoyl dithiocarbazinate, is approximately one-third as active as isonicotinoyl hydrazine, however, it is about one-third as toxic, so that the therapeutic index is approximately equal to that of isonicotinoyl hydrazine.

## EXPERIMENTAL

**3-(4-Pyridinecarboxamino)rhodanine (I).**—A solution of 13.7 Gm. (0.10 mole) of isonicotinoyl hydrazine in 100 cc. of water was placed into a 1,000-cc., round-bottomed flask provided with a reflux condenser. To the warm solution was added at once a solution of 22.6 Gm. (0.10 mole) of trithiocarbodiglycolic acid in 400 cc. of water. The mixture was heated on the steam bath for two and one-half hours and then cooled to room temperature. The yellow precipitate was removed by filtration with suction after twenty hours. The yield after drying *in vacuo* was 17.9 Gm. (70%). This crude product was purified by dissolving at 100° in 25 cc. of dimethylformamide, filtering and adding 25 cc. of water under vigorous stirring. After cooling with ice, filtering with suction, and drying *in vacuo* over calcium chloride, 14.0 Gm. (55%) of a slightly yellow product was obtained. The purification was repeated giving 12.8 Gm. (50%) of a product melting at 192–194° (decompd.) and darkening slowly at a lower temperature.

*Anal.*—Calcd. for $C_9H_7N_3O_2S_2$: C, 42.67; H, 2.78; N, 16.59; S, 25.32. Found: C, 43.04; H, 2.25; N, 16.56; S, 24.92.

The infrared spectrum was in agreement with the expected formula.

**Potassium 3-Isonicotinoyl Dithiocarbazinate (II).**—A solution of 13.7 Gm. (0.100 mole) of isonicotinoyl hydrazine in 100 cc. of absolute methanol was placed into a 250-cc., three-necked, round-bottomed flask provided with a stirrer, a reflux condenser, and a thermometer. Powdered anhydrous potassium carbonate (7.0 Gm.; 0.051 mole) and carbon disulfide (10.0 Gm.; 0.131 mole) were added. The mixture was stirred at 60° for four hours. The solid was removed by filtration with suction and was dried *in vacuo*. The yield was 20.0 Gm. (80%). The product was purified by adding 40 cc. of absolute ethanol to a clear solution of the product in 20 cc. of water. The white precipitate was removed by filtration and dried *in vacuo* over potassium hydroxide. The yield was 10.5 Gm. (42%).

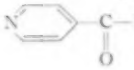
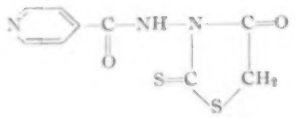
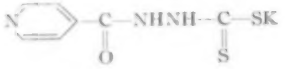
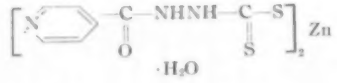
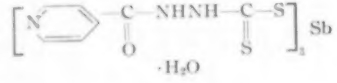
*Anal.*—Calcd. for $C_7H_6KN_3OS_2$: N, 16.72. Found: N, 16.48.

**Zinc 3-Isonicotinoyl Dithiocarbazinate.**—A solution of 6.0 Gm. (0.024 mole) of potassium 3-isonicotinoyl dithiocarbazinate in 20 cc. of distilled water was placed into a 100-cc. Erlenmeyer flask. A clear solution of 1.5 Gm. (0.007 mole) of zinc chloride in 20 cc. of distilled water and 5 cc. of 0.1 *N* hydrochloric acid was added dropwise with stirring to this solution. The yellow precipitate was filtered and washed successively with small portions of distilled water (total 50 cc.), ethanol (total 50 cc.), and ether. The product was dried *in vacuo*; yield 4.5 Gm.

*Anal.*—Calcd. for $C_{14}H_{12}N_6O_2S_4Zn \cdot 2H_2O$: Zn, 12.67. Found: Zn, 12.0.

**Antimony 3-Isonicotinoyl Dithiocarbazinate.**—A solution of 6.0 Gm. (0.024 mole) of potassium 3-isoni-

TABLE I.—MICE INFECTED INTRAVENOUSLY WITH *Mycobacterium tuberculosis*, H37Rv[a]

| Compounds | INH Equiv. Wt., % | MTD[b] Subc. | MTD[b] Oral | Drug Level mg./Kg./day | $\Delta ST_{50}$[c] or % Survival after 4 wks. Subc. | $\Delta ST_{50}$[c] or % Survival after 4 wks. Oral | MED[d] Subc. | MED[d] Oral |
|---|---|---|---|---|---|---|---|---|
|  INH | 100 | 150 | 100 | 5 | ... | 100 | | |
| | | | | 3 | 100 | | | |
| | | | | 1 | +10.0 | 80 | | |
| | | | | 0.5 | +4.0 | +3.0 | 0.5 | 0.5 |
| | | | | 0.25 | −1.0 | +1.0 | | |
|  | 93 | >500 | 400 | 500 | +5.5 | | >100 <500 | 400 |
| | | | | 400 | | +3.0 | | |
| | | | | 133 | | +1.0 | | |
| | | | | 100 | +2.0 | | | |
|  | 54 | >250 | >400 | 400 | | 100 | >1 <10 | >1 <10 |
| | | | | 250 | 100 | | | |
| | | | | 10 | +9.5 | +10.0 | | |
| | | | | 1 | 0 | −1.0 | | |
|  | 53 | | | 400 | | 100 | >1 <10 | >1 <10 |
| | | | | 250 | Toxic | | | |
| | | | | 200 | | 100 | | |
| | | | | 100 | 90[e] | | | |
| | | | | 50 | | 100 | | |
| | | | | 10 | 100 | 100 | | |
| | | | | 1 | −2.0 | −1.0 | | |
|  | 50 | | | 400 | | 90 | >1 <10 | >1 <10 |
| | | | | 250 | Toxic | | | |
| | | | | 200 | | 100 | | |
| | | | | 100 | 80[f] | | | |
| | | | | 50 | | 100 | | |
| | | | | 10 | 100 | 100 | | |
| | | | | 1 | −2.5 | −2.0 | | |

[a] Treated daily for ten days. Evaluated four weeks after infection started.
[b] MTD—Maximum tolerated dose.
[c] $\Delta ST_{50}$—Change median survival time of treated over untreated mice.
[d] MED—Minimum effective dose. The minimum amount of drug in mg./Kg./day which affords a $\Delta ST_{50}$ of 3.0 to 4.0 days. This increase of survival time is outside the standard error of 2.0 days.
[e] One mouse died of toxicity.
[f] Two mice died of toxicity.

cotinoyl dithiocarbazinate in 40 cc. of water was placed into a 100-cc. Erlenmeyer flask. A clear solution of 1.5 Gm. (0.007 mole) of antimony chloride in 5 cc. of concentrated hydrochloric acid was added dropwise with stirring. The orange-yellow precipitate was removed by filtration with suction and was washed successively with small portions of alcohol (total 50 cc.) and ether. The orange-yellow compound was dried *in vacuo;* yield 4.0 Gm.

*Anal.*—Calcd. for $C_{21}H_{18}N_9O_3S_6Sb.3H_2O$: Sb, 14.98. Found: Sb, 14.4.

**Therapeutic Acitivity in Mice Infected with *Mycobacterium tuberculosis* H37Rv.**—The biological tests were performed as outlined briefly below, applying Litchfield's method for determining median survival times (9).

Specifications of the materials: Four-to-six-weeks-old male albino mice of the CF No. 1 strain weighing 16–18 Gm. were used. The inoculum of *Mycobacterium tuberculosis* var. *hominis* (H37Rv) was prepared from infected mice lungs onto Loewenstein slants stored at 4°. The first subculture from the slant to modified Kirschner's medium with 0.02% Tween 80 and 2% bovine serum albumin was grown for seven days at 37°. Later subcultures in Kirschner's medium were grown for four days. The third subculture was taken for inoculation and 0.5 cc. was injected intravenously in mice. Viable counts showed that mice received about $10^6$ organisms.

Treatment: Three hours after infection, drug treatment was started. The drug, dissolved in water, or if insoluble in water, suspended in 50% Plazmoid,[1] was administered subcutaneously, or orally, daily for ten days using ten mice per group. Observation of the mice was discontinued after four weeks.

Evaluation: With the ten drug-treated mice, a series of tests were run with ten, infected, untreated mice parallel to the infected. The median survival time ($ST_{50}$) was determined by plotting the deaths in cumulative per cent as probits against days of survival as log. The change in the increased median survival time ($\Delta ST_{50}$) was expressed in days of survival of treated mice over untreated mice, thus $\Delta ST_{50}$ of + 2.0 days means the treated animals died an average of 2.0 days later than the untreated control group. $\Delta ST_{50}$ of −2.0 days means the treated animals died an average of 2.0 days sooner than the untreated control group.[2] If, after four weeks, more than 50% of the mice were still alive, the results were expressed in per cent survival instead of in increased median survival time ($\Delta ST_{50}$). The results of the experiments are shown in Table I. From this table one will notice that zinc and antimony 3-isonicotinoyl dithiocarbazinate cured all mice at levels as low as 10 mg./Kg./day.

[1] Product of The Upjohn Company, Kalamazoo, Mich. Trademark Reg. U. S. Pat. Off.

## SUMMARY

1. The preparation of four sulfur-containing isonicotinic acid hydrazide derivatives has been described; they are potassium, zinc, and antimony 3-isonicotinoyl dithiocarbazinate, and 3-(4-pyridinecarboxamino)rhodanine.

2. These compounds have been evaluated in mice against *Mycobacterium tuberculosis*, H37Rv, and their activity is compared with isonicotinoyl hydrazine. None of the compounds seems to be superior to the standard, isonicotinoyl hydrazine.

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[2] A statistical analysis of data obtained in this test system has revealed that the standard error of a $\Delta ST_{50}$ determination is 2.0 days.

# A Colorimetric Procedure for Imidazolines*

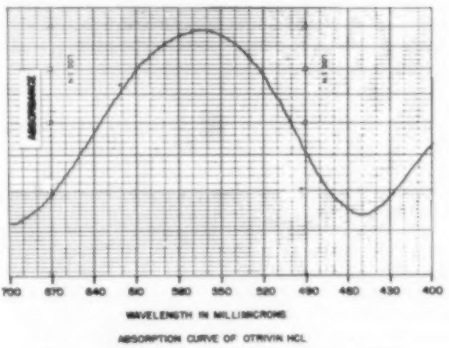
By S. C. SLACK and W. J. MADER

**Laubie's method for the identification of imidazolines has been found applicable to the quantitative estimation of 2-anilinomethyl-2-imidazoline hydrochloride (Otrivin® hydrochloride) and other substituted imidazolines in nasal solutions and tablets by treatment with sodium nitroprusside in alkaline solution to produce a violet color which obeys Beer's law within the ranges indicated. Optimum conditions for this color reaction and measurement have been defined.**

THE USE OF IMIDAZOLINES in combination with other therapeutic agents in pharmaceutical preparations illustrates the need for a rapid, specific assay procedure. A general identification test for imidazolines was proposed by Laubie (1, 2) based upon the treatment of the imidazoline with sodium nitroprusside solution in the presence of sodium hydroxide and subsequent addition of sodium bicarbonate to develop a violet color. This test was later modified by Laubie (3) to utilize hydrogen peroxide to accelerate oxidation and color development. The colorimetric procedure of Mader, Sterne, Rosin, and Frediani, for the determination of histamine is applicable to Priscoline® hydrochloride, and certain other imidazolines though it lacks sensitivity in the case of Otrivin hydrochloride. The ultraviolet absorption spectrum of Otrivin hydrochloride in methanol lends itself readily to the determination of this compound. However, for preparations containing interfering substances which cannot be separated by differential extraction, it was deemed advisable to develop a rapid procedure that embodies the requisite sensitivity and precision.

## EXPERIMENTAL

The general colorimetric identity reaction of Laubie for imidazolines has been modified to give quantitative results with 2-anilinomethyl-2-imidazoline hydrochloride (Otrivin hydrochloride); Antazoline hydrochloride, U. S. P. (Antistine hydrochloride); tolazoline hydrochloride, U. S. P. (Priscoline hydrochloride); and naphazoline hydrochloride, N. F. (Privine® hydrochloride). Difficulty was experienced in obtaining a stable color with phentolamine hydrochloride, U. S. P. (Regitine® hydrochloride). In this method, the imidazoline is treated with sodium nitroprusside in an aqueous sodium hydroxide solution. Addition of an excess of sodium bicarbonate solution results in the formation of a stable violet color with an absorption maximum at 565 mμ. With Otrivin hydrochloride, the color follows Beer's law in the concentration range of 0–0.5 mg. The method is rapid and reproducible (standard deviation ±2%). Aldehydes and certain other compounds which react with sodium nitroprusside in alkaline solution interfere, but their presence is not encountered in the usual imidazoline preparations.




ABSORPTION CURVE OF OTRIVIN HCL

Fig. 1.—Absorption curve for Otrivin HCl.

## REAGENTS

Five per cent sodium nitroprusside: 5.0 grams of reagent grade crystals are dissolved in water and diluted to 100 ml.

One molar sodium hydroxide: Dissolve 4.0 grams of reagent grade sodium hydroxide pellets in water and dilute to 100 ml.

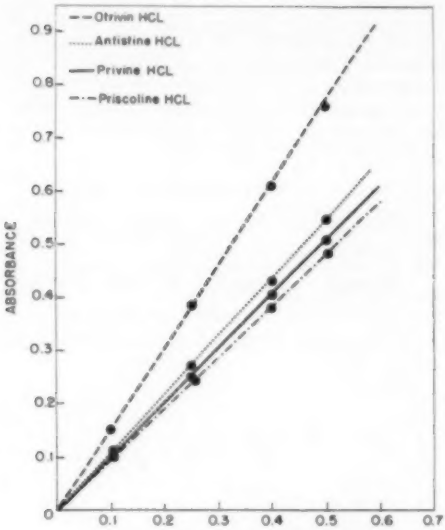
One molar sodium bicarbonate: 8.4 grams of reagent grade sodium bicarbonate is dissolved in water and diluted to 100 ml.

Standard imidazoline solution: To prepare standard solutions of Antistine, Privine, and Priscoline hydrochlorides, dissolve 50.0 mg. of the salt in water and dilute to 100 ml. In the case of Otrivin hydrochloride, dissolve 25.0 mg. of the salt in water and dilute to 100 ml.

## PROCEDURE

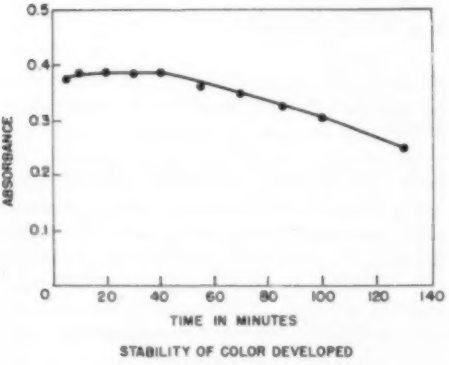
Dilute the solution to be assayed so that 1.0 ml. contains an amount theoretically equivalent to the concentration of the standard solution. To one of three 10-ml. volumetric flasks, add 1.0 ml. of distilled water; to the second, 1.0 ml. of standard solution; and to the third, 1.0 ml. of the solution to be assayed. Add 0.5 ml. of sodium hydroxide solution to each flask, followed by 0.5 ml. of sodium nitroprusside solution. Mix well and set aside for ten minutes. Add 1.0 ml. of sodium bicarbonate solution to each flask and make up to volume with distilled water. Mix and set aside for ten minutes. Measure the absorbance values of both the standard and the

* Received May 3, 1957, from the Research Department, CIBA Pharmaceutical Products Inc., Summit, N. J.
Presented to the Scientific Section, A. PH. A., New York meeting, May 1957.



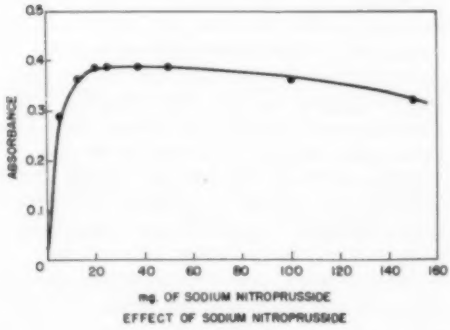

ADHERENCE TO BEER'S LAW

Figure 2.



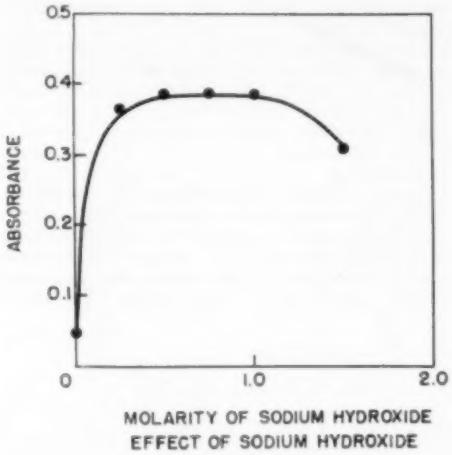

STABILITY OF COLOR DEVELOPED

Figure 3.




EFFECT OF SODIUM NITROPRUSSIDE

Figure 4.




EFFECT OF SODIUM HYDROXIDE

Figure 5.

solution for assay against the reagent blank at 565 mμ in a suitable spectrophotometer, using matched, one-centimeter cells. The violet color produced is stable for thirty minutes in the case of Otrivin hydrochloride. The concentration of the solution assayed is calculated as follows:

$$[A \text{ (sample)}]/[A \text{ (standard)}] \times \text{concn. standard (mg./ml.)} = \text{concn. sample (mg./ml.)}$$

Figure 1 shows an absorption curve obtained with a Beckman DK recording spectrophotometer for Otrivin hydrochloride, indicating a maximum at 565 mμ and a minimum at 450 mμ. The absorption curves of the other imidazolines tested are similar to that obtained for Otrivin hydrochloride.

## DISCUSSION

Figure 2 shows the adherence to Beer's law for the colors produced with Otrivin hydrochloride, Antistine hydrochloride, Privine hydrochloride, and Priscoline hydrochloride. The method has been successfully applied to nasal solutions and to tablets, after extraction of the imidazoline, with standard deviations of ±2%. Nasal solutions containing Otrivin hydrochloride assayed by ultraviolet absorption techniques give results which showed good agreement with those obtained by the colorimetric procedure.

## STUDY OF VARIABLES

**Effect of Time.**—This effect was studied by treating a solution of Otrivin hydrochloride containing 0.25 mg./ml. by the recommended procedure and determining the optical characteristics at 565 mμ of the solution at different time intervals. Figure 3 shows that maximum intensity was obtained after ten minutes and was constant for an additional thirty minutes.

**Effect of Sodium Nitroprusside.**—A plot of absorbance *vs.* mg. of sodium nitroprusside at 565 mμ (Fig. 4) shows that maximum intensity is obtained when a volume of reagent containing 20–50

mg. (0.4–1.0 ml. of a 5% solution) of sodium nitroprusside is used.

**Effect of Sodium Hydroxide.**—Figure 5 illustrates the effect of the molarity of the sodium hydroxide solution on color intensity. Maximum intensity is obtained when the sodium hydroxide concentration is 0.5–1.0 molar. However, maximum stability of color is obtained when 0.5 ml. of a one-molar solution is used.

**Effect of Sodium Bicarbonate.**—An amount of sodium bicarbonate greater than that of the sodium hydroxide present is necessary for full development of color. When solid sodium bicarbonate is added in place of sodium bicarbonate solution, the violet color changes to rose and fades rapidly. For maximum color stability, 1.0 ml. of one-molar solution was found to give the best results in the case of Otrivin hydrochloride. However, optimum conditions should be studied for each imidazoline under consideration. A volume up to 2.0 ml. of a one-molar sodium bicarbonate solution does not affect color intensity, but greater volumes result in less intense and less stable solutions.

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# Reaction of Rauwolfia Alkaloids, Indoles, and Related Compounds to Nitrite*

By R. P. HAYCOCK and W. J. MADER

**The greenish-yellow color complex resulting from the addition of sodium nitrite to an alcoholic solution of reserpine in the presence of dilute sulfuric acid has been re-examined to determine the selectivity of the reaction and the chromophore groups fundamentally responsible for the color. Its application to other rauwolfia alkaloids, indoles, and related compounds was investigated. A new indican reaction is reported for simpler indoles.**

THE RAUWOLFIA ALKALOIDS have received much attention since the isolation of reserpine was reported by Mueller, Schlittler, and Bein (1). The difficulties involved in establishing a method of analysis specific for reserpine is apparent when one considers the structural complexity of reserpine and its similarity to other alkaloids found in various species of Rauwolfia. Without enumerating all procedures, mention should be made of some of the methods described for the separation and determination of reserpine in therapeutic preparations. Chromatographic techniques, both paper and column, have been used extensively (2–4). A method is described for the estimation of reserpine based on the ultraviolet absorption of 3,4,5-trimethoxybenzoic acid liberated by hydrolysis (5). A fluorometric method for the determination of reserpine in tablets and extracts of *Rauwolfia serpentina* is based on a reaction with hydrogen peroxide (6). Reserpine has been determined by ultraviolet absorption and colorimetrically and fluorometrically after heating with strong acids (7). Other colorimetric procedures include: the formation of a chloroform soluble complex with bromophenol blue (8), a color complex using a conventional reagent, vanillin (9), and a colorimetric technique using sodium nitrite in the presence of sulfuric acid (10).

Notwithstanding the abundance of analytical procedures, the application of existing methods presented certain difficulties and inconveniences. Consequently, the colorimetric Szalkowski-Mader nitrite technique was re-examined. The authors indicate that the greenish-yellow colored complex resulting from the addition of sodium nitrite to a methanolic solution of reserpine in the presence of sulfuric acid is specific and does not react with alstonine, rauwolscine, sarpagine, yohimbine, and deserpidine. Although no fundamental changes were made, Banes (11, 12) and coworkers proposed improvements to the application of the method to reserpine and to reserpine-rescinnamine group alkaloids in pharmaceutical

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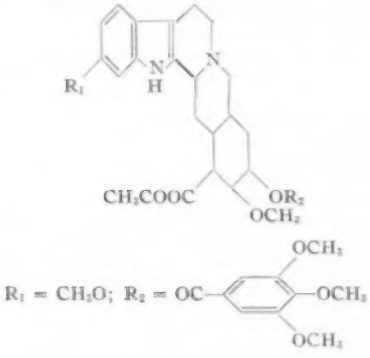
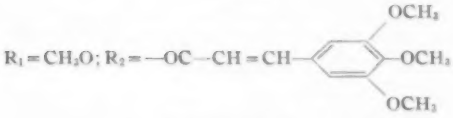
Presented to the Scientific Section, A. PH. A., New York, meeting, May 1957.

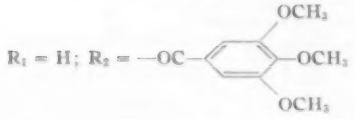
preparations. This modification, according to Banes, corrects for 3-dehydroreserpine a degradation product of reserpine and other substances absorbing light at 390 mμ prior to nitrite treatment. However, the selectivity of the reaction and the chromophore groups fundamentally responsible for the color has not been adequately investigated. This led to the study of a number of alkaloids, indoles, and related compounds, to determine the effect of variations in the molecule upon the colorimetric nitrite procedure, on the assumption that this might find application in the elucidation, or corroboration, of the structure of alkaloids from various species of Rauwolfia.




Reserpine




Rescinnamine




Deserpidine

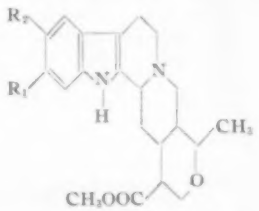
Figure 1.

## EXPERIMENTAL

**Solutions.**—(*a*) Indole solutions: Dissolve 25.0 mg. of the indole in 100 ml. of methanol. Dilute 4.0 ml. to exactly 100 ml. with methanol. The diluted indole solutions contain 10 mcg. of the indole per ml. (*b*) Carboline solutions: Dissolve 5.0 mg. of the carboline in 100 ml. of methanol. Dilute 10.0 ml. to exactly 100 ml. with methanol. The diluted carboline solutions contain 5 mcg. of the carboline per ml. (*c*) Alkaloid solutions: Dissolve 5.0 mg. of the alkaloid in 100 ml. of methanol. Dilute 20 ml. to exactly 50 ml. with methanol. The diluted alkaloid solutions contain 20 mcg. of the alkaloid per ml. (*d*) Sulfuric acid solutions 1 *N*: Dissolve about 30 ml. of sulfuric acid in one liter of water. (*e*) Aqueous sodium nitrite solution 0.3%. (*f*) Aqueous sulfamic acid solution, 5%. Prepare fresh every two or three days.

## PROCEDURE

The procedure is essentially that of Szalkowski and Mader (10) as modified by Banes and associates (11). Transfer duplicate 10.0-ml. aliquots equivalent to 100 mcg. of the indoles, 50 mcg. of the carbolines, and 200 mcg. of the alkaloids to 150 x 18 mm. test tubes. To each tube add 3.0 ml. of sulfuric



$R_1 = CH_3O$; $R_2 = H$
Reserpinine

$R_1 = CH_3O$; $R_2 = CH_3O$
Reserpiline

$R_1 = H$; $R_2 = CH_3O$
Aricine

Figure 2.

acid solution, to one of the duplicate tubes add 2.0 ml. of sodium nitrite solution, and to the other duplicate tube add 2.0 ml. of distilled water. Mix the contents of each tube and allow to stand for one hour. Add to each tube 0.5 ml. of sulfamic acid solution, mix, and filter through paper. After stabilization of the solutions, determine the spectral characteristics with a suitable recording spectrophotometer in matched 1-cm. cuvettes relative to the sample blank containing no nitrite.

## DISCUSSION

As indicated previously, the authors of the nitrite procedure for reserpine claimed that sarpagine, rauwolscine, alstonine, yohimbine, and deserpidine, did not react. An examination of the structure of these alkaloids indicated that the common structural difference from reserpine was the absence of an 11-methoxy group. Consequently, the spectral characteristics of other tertiary indole alkaloids were investigated to verify these observations. Rescinnamine (Fig. 1), which differs from reserpine only in that the esterfied alcoholic function is trimethoxycinnamic acid, instead of trimethoxybenzoic acid, produced chromogenic values approximately equal to those of reserpine. Reserpinine (Fig. 2), a tertiary indole with a heterocyclic ring *E*, and possessing an 11-methoxy group, also formed the greenish-yellow complex. In order to determine the effect of two methoxy groups in ring *A*, reserpiline, which possesses a 10,11-dimethoxy group, was examined. The color complex showed essentially

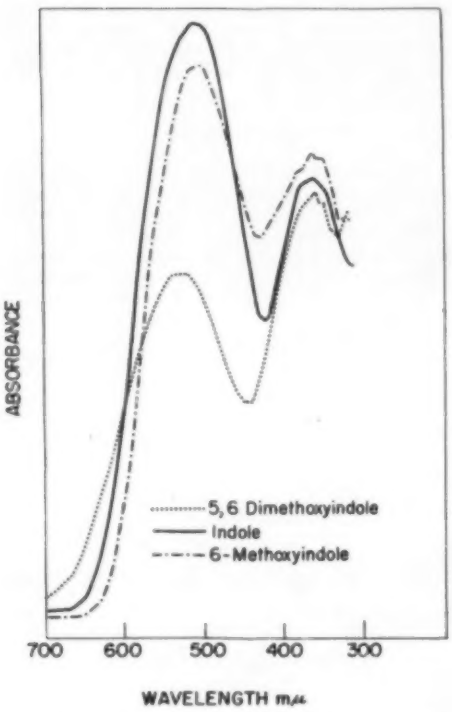



Fig. 3.—Absorption curves of nitrite treated indoles.

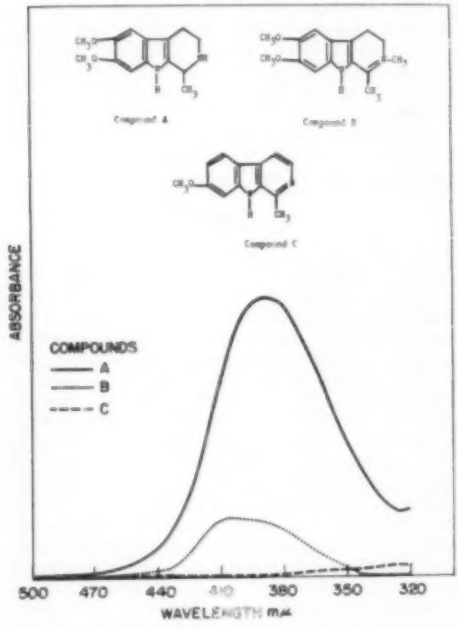
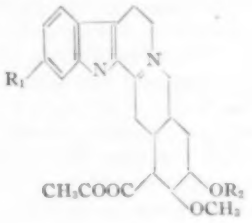
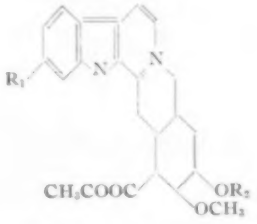



Fig. 4.—Absorption curves of nitrite treated carbolines.

the absorption of reserpinine. Aricine, which differs from reserpinine only in the position of the methoxy group, which in this alkaloid is in the 10 position, does not react. It is of interest to note that aricine develops a weak reaction on




3 Dehydroreserpine




Tetra Dehydroreserpine

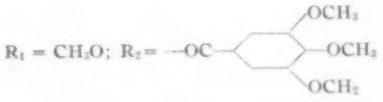



Figure 5.

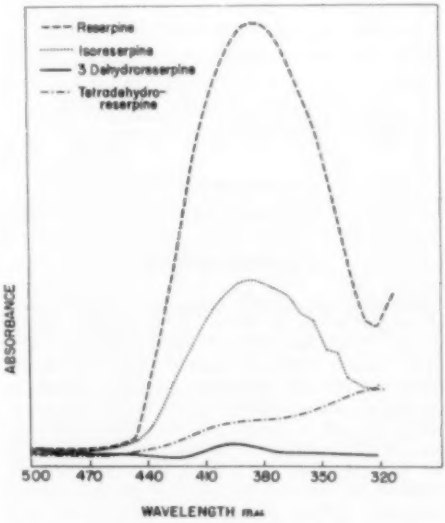



Fig 6.—Absorption curves of nitrite treated reserpine and degration products.

standing several hours. Raunescine, which also lacks an 11-methoxy group, did not yield the characteristic nitrite color. It appeared probable from the before mentioned data that an 11-methoxy group was one of the underlying groups necessary in the development of the greenish-yellow nitrite color.

In order to determine other groups necessary for the reaction, a number of indoles and related compounds were studied. When the behavior of indoles was examined as shown in Fig. 3, it was found that the addition of dilute sulfuric acid-sodium nitrite reagent to an alcoholic solution of indole, 6-methoxyindole, and 5,6-dimethoxyindole produced a pink color, rather than the greenish-yellow colow characteristic of reserpine. The effect of the concentrations of sulfuric acid and sodium nitrite, and the effect of time and temperature on the intensity and stability of the pink colored complex was not examined. However, the color appears to be stable and presumably it is a new indican reaction. The addition of groups at the 2-position reduces the color intensity displayed by the other indoles unsubstituted in that position.

Finally, the nitrite spectra of a series of β-carbolines was examined. These results are shown in Fig. 4. Compound *A*, 1,2,3,4-tetrahydro-6,7-dimethoxy-1-methylβ-carboline, which is similar to the *ABC* ring skeleton of reserpine, shows the characteristic spectrum of nitrite treated reserpine with a maximum at 390 mμ. However, compounds *B* and *C* or 6,7-dimethyl-1-methyl-3,4-dihydrocarboline and harmine which contain unsaturation in ring *C*, did not form the greenish-yellow color phenomenon. This led to the conclusion that the *ABC* ring structure of reserpine is a necessary adjunct to the formation of the color complex.

Although Banes (12) and co-workers observed that reserpine could be determined in the presence of its oxidative degradation products, no data was reported on the application of the nitrite procedure to pure 3-dehydroreserpine and tetradehydroreserpine (Fig. 5). This suggested a further study of these oxidative degradation products to ascertain whether or not the color phenomenon develops upon treatment with nitrite reagent. An examination of the absorption obtained by treating alcoholic solutions of 3-dehydroreserpine and tetradehydroreserpine with nitrite is negative and is shown in Fig. 6. This is essentially in agreement with the negative results obtained with the unsaturated β-carbolines noted previously, and substantiates the observation of Banes that reserpine can be determined in the presence of oxidation products by the nitrite procedure. Isoreserpine, which is also relatively inactive, exhibits chromogenic absorbance but it is considerably less than that of reserpine as can be seen in Fig. 6.

In earlier attempts to reproduce the spectral curves for nitrite treated reserpine, and other alkaloids, we noted a peculiar absorption pattern in the range 330–380 mμ unlike that reported by Szalkowski and Mader. Subsequent investigation revealed that this irregular absorption resulted from excess sodium nitrite as indicated in Fig. 7. Improved results were obtained by filtering the reaction mixture through filter paper, but in the case of nonreactive alkaloids and indoles, the addition of sulfamic acid directly to the reaction medium is required to remove excess nitrite and eliminate misleading results.

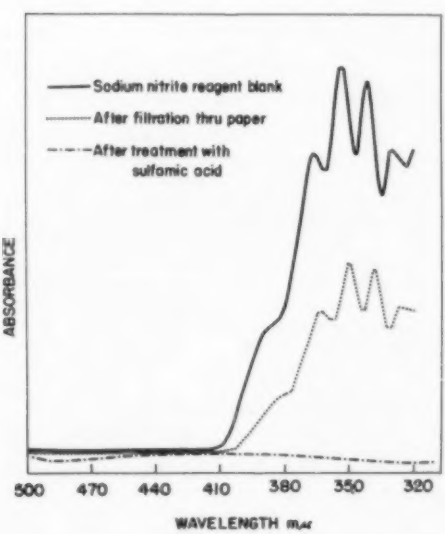



Fig. 7.—Absorption curves of sodium nitrite.

## CONCLUSIONS

1. It has been established that the 7-methoxy β-carboline group, i. e., the 11-methoxy group and the *AB* and *C* ring skeleton of reserpine, is the functional group responsible for the greenish-yellow complex obtained by reacting an alcoholic solution of reserpine with sodium nitrite in the presence of dilute sulfuric acid.

2. On the basis of the formation of a greenish-yellow color with nitrite, the presence of an 11-methoxy group in alkaloids may be ascertained.

3. On the basis of sensitivity, selectivity, and reproducibility, the nitrite technique is the preferred procedure for determining reserpine.

4. A new indican reaction is reported for some indoles.

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(12) Banes, D., Wolff, J., Fallscheer, H. O., and Carol, J. *ibid.*, **45**, 710(1956).

# Book Notices

*Mathematics and Statistics for Use in Pharmacy, Biology, and Chemistry.* By L. SAUNDERS and R. FLEMING. The Pharmaceutical Press, London, 1957. x + 257 pp. 13.5 x 22.5 cm. Price 27*s.*, 6*d.*

This book is designed as a short course in mathematics and statistics which can be utilized by those who do not have advanced knowledge of either subject. The design is good, although the absence of descriptive prose might cause an erroneous first impression that the book is too highly technical for the intended readers, which include students in the fields of pharmacy (particularly in courses of analysis—chemical and biological), medicine, biology, and chemistry, and graduate students interested in statistical applications.

The text begins with chapters devoted to arithmetic, algebra, graphs, logarithms, and simple calculus. These are followed by trigonometry, more advanced calculus, probability, statistical methods, and the final two chapters on applications of statistics to biological assays, bacteriology, and pharmaceutical practices. Particularly interesting is the treatment of "Dispensing errors." The authors do not approve the elimination of samples showing extremely wide deviations (which are obviously caused by blunders in calculations or misunderstanding on the part of some compounders) as Goldstein has done [see THIS JOURNAL, **38,** 131(1949)] in determining permissible limits of deviations in compounding operations, "since this introduces the pernicious principle of selecting results for statistical calculation." Goldstein utilized standard deviations and the 1.73 factor developed by Peters and VanVoorhis as a "working ratio" for provisional acceptance of the findings of an experiment. It might be inferred that Saunders and Fleming would use all the collected and tested samples to determine suitable tolerance limits; however, the authors state: "The straightforward calculation of standard deviation does, however, appear to give undue weight to these widely deviating results, and a better estimate of random dispensing error as is required for fixing tolerance limits for the future can be made by considering the proportion of all the results within certain limits of the prescribed value. Such a calculation can only be made when, as in the present case, a large number of results are available. The limits should be chosen so as to include about 95 per cent of the results, corresponding to the usual probability level for limits of error." It is also interesting to note that the example used in the text—merely dispensing 60 minims of liquefied phenol—yields limits of ±20 per cent to include 95 per cent of the 102 samples. This will probably surprise some of our British and U. S. friends who criticized the generosity of Goldstein's determined tolerances of ±10 per cent to ±17.5 per cent for extemporaneously compounded liquid preparations [see THIS JOURNAL, **40,** 186(1951)].

The last 47 pages of the book are devoted to 13 appendixes which include tables of functions and formulas, proofs of some of the quoted theorems, four-figure logarithms and antilogarithms, and answers to problems given in the text. The appended subject index makes this useful textbook a good reference and refresher aid for students, teachers, and practitioners.—S. W. GOLDSTEIN.

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